Human Hemicornea Model for Drug Transport Testing and Screening of Excipients

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Este trabajo se lo dedico a mi familia.

A little more persistence, a little more effort,
and what seemed hopeless failure
may turn to glorious success.
Elbert Hubbard

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Table of contents

Definitions and Abbreviations

(Å)	Ångström
α	Alfa
ß	Beta
x_i	Set of numbers
$\bar{x}$	Mean value or average
µCi	Microcurie
µg	Microgram
µL	Microliter
µm	Micrometer
3D	3–dimensional
A	Filter growth area
a	Apical
ab	Apical to basolateral transport
ACB	Across Barriers GmbH
ALI	Air liquid interface or Air liquid culture conditions
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
b	Basal or basolateral
ba	Basolateral to apical transport
BAC	Benzalkonium chloride
BfArM	German Federal Institute for Drugs and Medical Devices
BCRP	Breast cancer resistance protein
BCS	Biopharmaceutics classifications system
Caco-2 cells	Human colon adenocarcinoma cell line
Calu-3 cells	ATCC cell line HT-55
Ce	Cetrimide
CEPI-17-CL 4	Corneal epithelial cell line
CD	Chlorhexidine digluconate
cm	Centimeter
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpm	Decays per minute or disintegrations per minute
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FD-4	Fluorescein Isothiocyanate–dextran

g	Gram
HBSS	Hanks' Balanced Salt Solution
HCE	Human corneal epithelial cell line 10.014 pRSV-T
HCE-T	Human corneal epithelial cell line
HCK	Human corneal keratocyte cell line
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
K	Keratin
Ka	Acid dissociation constant
KBM	Keratinocyte basal medium
kDa	Kilodalton
KGM	KBM + Single Quotes (Keratinocyte growth medium)
KRB	Krebs-Ringer bicarbonate-buffer
L	Liter
LCC	Liquid covered condition
log *D*	Logarithmic distribution coefficient
log *P*	Logarithmic partition coefficient
LRP	Lung resistance-related protein
Ltd	Limited
MDCK	Madin-Darby canine kidney
Min	Minutes
MRP	Multidrug resistance associated protein
mL	Milliliter
mm	Millimeter
mM	Milimolar
MP	Methylparaben
mRNA	Messenger ribonucleic acid
MW	Molecular weight
n	Number of repeats within an experiment
Na-FLU	Sodium fluorescein
P_{app}	Apparent permeability coefficient
PEG	Polyethylene glycols oligomer
PBS	Phosphate buffered saline
P-gp or MDR1	P-glycoprotein
PQ-1	Polyquaternium-1 or Polyquad®
pRCE	Primary rabbit corneal epithelial cells
R	Rabbit
Ref	Reference

RCE	Rabbit corneal epithelial cell line
rpm	Revolutions per minute
RSD	Relative standard deviation
SD	Standard deviation
RCE	Rabbit corneal epithelial cell line
SIRC	Statens Seruminstitut Rabbit Cornea
SV40	Simian Virus 40
TEER	Transepithelial electrical resistance
Thio	Thiomersal
TJs	Tight junctions
TUBS	Technische Universität Braunschweig, Institut für Pharmazeutische Technologie, Braunschweig, Germany
UKE	Department of Dermatology and Venereology of the University Hospital Hamburg-Eppendorf, Germany
USP	United States Pharmacopeia
ZO	Zona occludens

1. Introduction

During drug development alternative *in vitro* cell culture models are becoming more and more important in order to reduce or replace *in vivo* or *ex vivo* animal experiments, in particular in the preclinical phase. Compared to *in vivo* / *ex vivo* experiments, *in vitro* cell culture models offer many advantages since the experimental conditions are easier to control and the reproducibility is usually higher. Furthermore, cell culture models are also easier to standardize, more cost-effective and a constant and predictable source of research material for continuous investigations.

In the development of new ophthalmic medications or related studies conducted to define their bioequivalence, transcorneal permeation studies are usually carried out *in vivo* or using *ex vivo* animal cornea. Due to the poor availability of *ex vivo* human corneas, corneal tissue of rabbit origin is commonly used in such studies. Over the last 20 years, a variety of different *in vitro* cell culture based corneal models have been developed for drug permeation studies. However, despite the considerable progress made in this area of research, these *in vitro* corneal models also have disadvantages and aspects that need to be further evaluated and improved before they are suitable for wide application in the pharmaceutical industry and within the scope of regulatory processes. The disadvantages of these alternative models include, for instance, the requirement for validation, the requirement to fulfill good laboratory practice (GLP) regulations, the analysis of comparability of data to those achieved with isolated cornea regarding a wide range of substances with different physicochemical properties as well as ophthalmic formulations, and the proof of transferability of results to humans.

Since many questions remain unclear in this context, which restricts the widespread use of these corneal models, the aims of the present study were, first, to perform and assess the transfer of standard operating procedures for the cultivation of an *in vitro* corneal model and its use for drug permeability studies from an academic research laboratory to a biotech laboratory (Across Barriers GmbH; ACB); secondly, to participate in a prevalidation project including permeation data obtained with the corneal model at ACB; and thirdly, to evaluate the usefulness of the corneal model in *in vitro* permeation studies with a wide range of substances and excipients for ophthalmic formulations. The corneal model that has been used for the current study is a three-dimensional human Hemicornea model (Hemicornea; HC), also known as Hemicornea construct, that has been developed by the Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Germany (TUBS) (Hahne and Reichl*,* 2011). The present study intended to prove the suitability of the HC construct as *in vitro* model for preclinical permeability studies during the development of new ophthalmic medications, and to answer the following questions:

1. Is it possible to transfer the standard operating procedures for the cultivation of the HC construct and drug permeability studies to an industrial laboratory, and what acceptance criteria need to be defined for the application of the HC construct?

   Does the HC construct possess an acceptable degree of intra- and interlaboratory reproducibility in permeability studies and can the HC construct be prevalidated for *in vitro* drug permeability studies?

2. Are the barrier properties and permeability (P_{app}) of the HC construct with regard to drugs and marker substances with a wide range of molecular attributes comparable to those reported for *ex vivo* rabbit and human corneal tissue?

3. To what extent are the epithelial integrity and permeability of the HC construct influenced by physicochemical parameters of ophthalmic formulations, such as pH and osmolality?

4. Is the permeability of the HC construct influenced by excipients commonly used in ophthalmic medications for topical application to the eye, such as EDTA and preservatives, and are the data obtained in these studies with the HC construct comparable to those reported for *ex vivo* corneal tissue?

2. Literature review

2.1. Anatomy of the eye

The human eye is one of five human sensory organs. The visible light is detected by the human eye and is transformed into electro-chemical signals by the optic nerve and then sent to the brain. This mechanism helps humans to establish a connection with their physical environment. The ocular globe can be divided into the anterior compartment and the posterior compartment. A detailed description of the anatomy and physiology of the eye is provided below.

2.1.1. Anterior compartment

The anterior compartment of the eyeball is formed by the cornea, conjunctiva, aqueous humor, iris, ciliary body and crystalline lens (Figure 2-1). These structures constitute almost one-third of the front part of the eye (Cholkar *et al.,* 2013).

2.1.2. Posterior compartment

The posterior compartment is composed of the sclera, choroid, Bruch's membrane, retinal pigment epithelium, neural retina and vitreous humor (Figure 2-1).

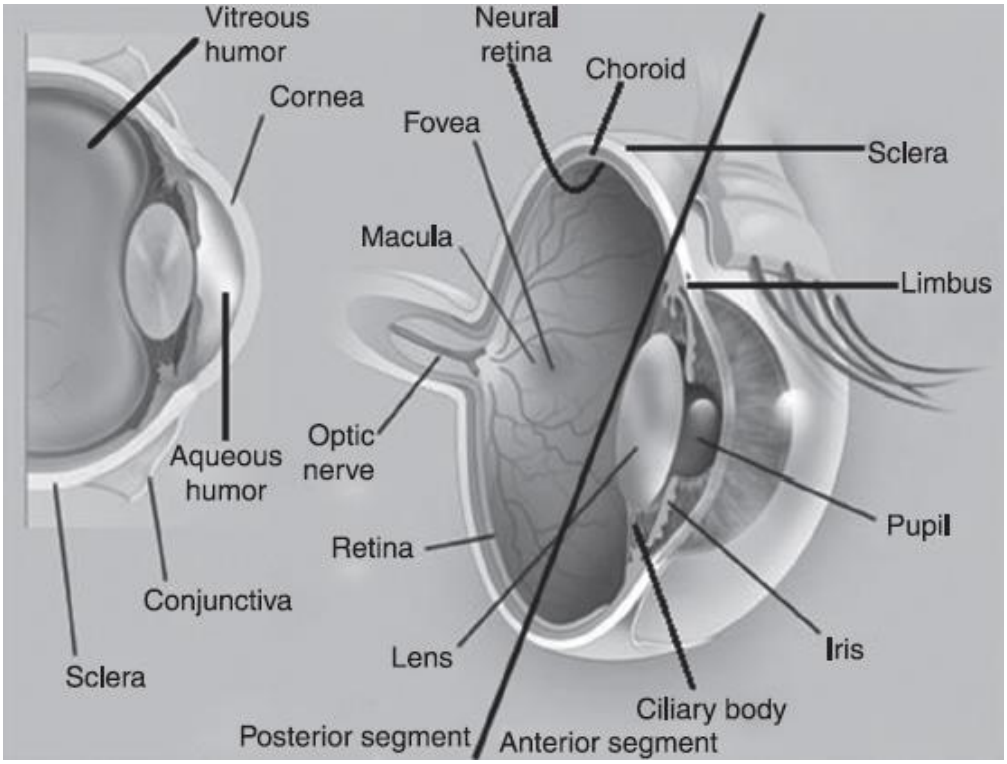

Figure 2-1: Representation of the anatomy of the human eye (Cholkar *et al.,* 2013)

2.2. Cornea

The cornea is defined as the window of the eye to the exterior environment. It is characterized by its transparency and regularity (Ehlers and Hjortdal, 2005). The human cornea has no blood vessels (non-vascular) and consists of three cellular layers: the corneal epithelium, the corneal stroma and the corneal endothelium, which are separated by two fine layers, the Bowman's layer and Descemet's membrane (Castro-Muñozledo, 2008).

The cornea's function is to facilitate the ability to see and to prevent external organisms from entering into the inter-neuronal membrane of the retina (Stepp, 2010). The disruption of the corneal barrier leads to ocular illness or ocular disorders and might lead to a loss of vision in the worst case (Lonsberry *et al.,* 2008). A schematic representation of the human cornea is shown in Figure 2-2.

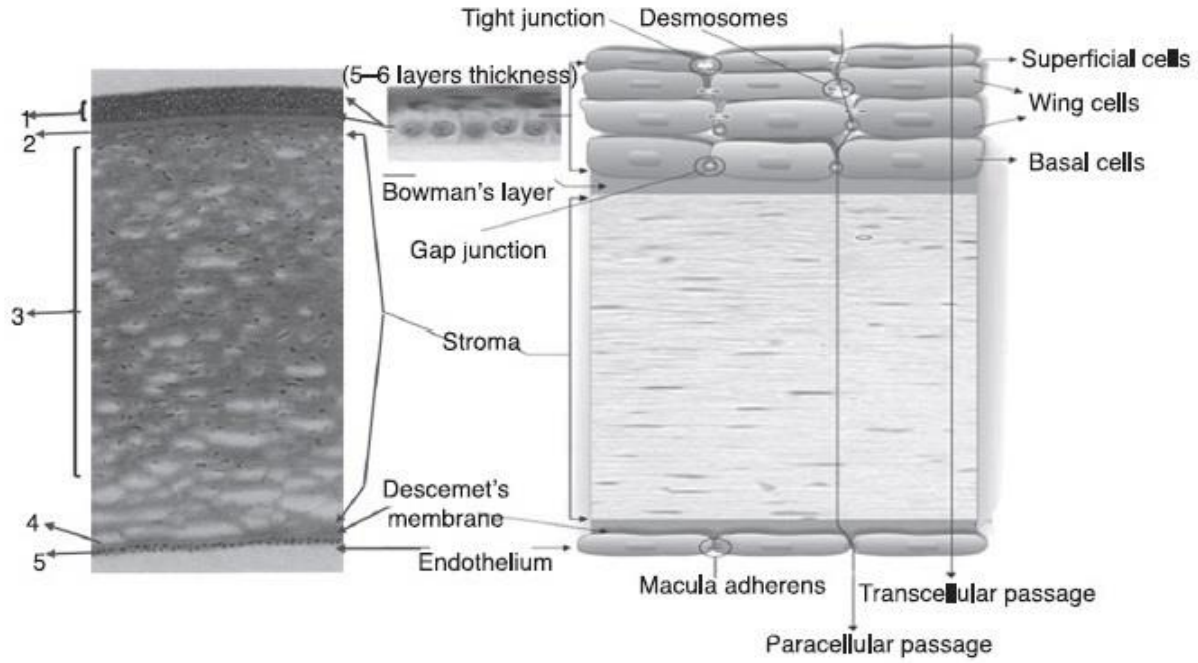

Figure 2-2: Schematic representation of the structure of the human cornea: 1. corneal epithelium (consists of superficial cells, wing cells, basal cells), 2. Bowman's layer, 3. corneal stroma, 4. Descemet's membrane and 5. corneal endothelium (adapted from Cholkar *et al.,* 2013)

2.2.1. Corneal epithelium

The corneal epithelium is made up of various squamous cell types, which differ in size and form. These squamous cells form a net structure (Lonsberry *et al.,* 2008). Moreover, the epithelium is composed of three layers of flattened cells, two to three layers of wing cells and one layer of columnar basal cells. The epithelium is highly innervated due to its large number of nervous connections (Huhtala *et al.,* 2008). The cells are divided in three compartments: the internal, intermediate and external epithelium. The internal epithelium consists of basal

cells, which are arranged in a columnar structure and closely packed. The intermediate part is built of two or three layers of wing cells. The external epithelium consists of two or three segments of squamous and flattened cells. A smooth and non-undulating basement membrane is located beneath the basal cells, which are attached to each other by hemidesmosomes. In addition, the basement membrane is a thin layer, which supports the corneal epithelium and has a thickness of approximately 40 - 60 nm (Ehlers and Hjortdal, 2005).

2.2.2. Bowman's layer

The Bowman's layer, also called Bowman's membrane, has a thickness of approximately 8 - 12 µm and consists of clear, randomly organized collagen fibrils (collagen type I and III) and proteoglycans (Reichl and Becker, 2008). This thin layer has been described as an extracellular matrix and impedes the direct epithelial-stromal interaction (Gabison *et al.,* 2009). Furthermore, it has been proposed that this membrane has a barrier function, which avoids the spreading of the herpex simplex and other viruses, which cause ocular diseases (Wilson and Hong, 2000). Moreover, it has also been suggested that this layer presumably acts as a biologic and physiological barrier (Obata and Tsuru, 2007). The rigidity, form and curvature of the anterior part of the corneal stroma are also attributed to the Bowman's layer (Morishige *et al.,* 2007).

2.2.3. Stroma

The thickness of the cornea is approximately 500 µm to 600 µm (Funderburgh, 2010). The major component of the cornea is the stroma, which constitutes about 90% of the whole tissue (Ehlers and Hjortdal, 2005). The corneal stroma consists of water (78%) and corneal fibroblasts (3-5%), also known as keratocytes, and an extracellular matrix, which is largely composed of collagen and glycosaminoglycans (Reichl and Becker, 2008). The stroma has about 71% collagen by dry weight (Newsome *et al.,* 1982).

The collagen fibrils have a width of between 10 µm and 250 µm, possess a thickness of between 200 nm to 250 nm and are arranged in parallel, forming lamellae. Approximately 258 to 290 lamellae are distributed in the stroma and lie parallel to the corneal surface, resulting in a total of 832 lamellae (Radner *et al.,* 1998; Radner and Mallinger, 2002). The keratocyte cells are flattened and lie between the lamellae. The keratocytes are randomly arranged and contribute to the cornea's stability even when the cornea is extensively inflamed (Müller *et al.,* 2001). The corneal fibroblasts synthesize the extracellular matrix, which basically consists of collagen (Poole *et al.,* 1993). The collagen possesses mainly a structural function. However, other collagen types act as chemotactic factors, antigens for the production of antibodies, cell attachment and cell differentiation. Collagen is considered to be

the protein, which is most present in the connective tissue (Knupp *et al.,* 2009). For instance, collagen type I is the most abundant in the corneal stroma, whereas collagen III and V have been found in pepsin extracts of human cornea (Newsome *et al.,* 1982). The presence of collagen IV in stromal tissue was confirmed by immunochemistry experiments (Marshall *et al.,* 1990). The transparency of the cornea has been discussed for years (Funderburgh, 2010). A mathematical model has been developed to support the assumption that the stromal transparency is the result of the structure of its collagen fibrils (Farrell *et al.,* 1973). In addition, the specific arrangement of the lamellae is not random (McCally and Farrell, 1982). The most important factor for conserving the corneal transparency may be the degree of stromal hydration. The discovery of a new protein (crystallins) in the keratocytes and other types of cellular tissue of the cornea suggested that these proteins serve to alter the refractive index of cells. For this reason, the scattered light is diminished by corneal cells (Funderburgh, 2010).

2.2.4. Descemet's membrane

The corneal endothelium's basement membrane is named Descemet's membrane. After birth, its thickness is almost 2.8 µm. The Descemet's membrane does neither possess a cellular structure nor an internal structure (Doshi, 2004). The expression of collagen IV was found in bovine cornea (Tseng *et al.,* 1982). Corneas from humans at different ages were observed under an electron microscopy; the data indicated that fibers form a bridge between the innermost parts of Descemet's membrane and might provide stability to the eye under mechanical stress (Binder *et al.,* 1991).

2.2.5. Corneal endothelium

The corneal endothelium is formed by polygonal cells. A high proportion of these cells are flattened and hexagonal; each individual cell has a width of 18 µm to 20 µm and a thickness of 5.0 µm (Whikehart, 2010). The main function of the endothelium layers is to permit aqueous humor to enter in a controlled manner in order to regulate the corneal transparency. If this equilibrium is broken, the homeostasis could be disrupted. The endothelium has a higher permeability than the epithelium because it controls the transport of substances supplying the stroma and epithelium with nutrients. This transport is regulated by the gap junctions and the tight junctions on the upper side of the endothelium (Mergler and Pleyer, 2007). The occludin and zona occludens (ZO-1) are located on the apical side of the endothelium (Joyce, 2003). The endothelial cell layer contains ca. 3000 cells·mm^{-2}. The number of cells decreases with age (Whikehart, 2010) since the endothelial cells are not able to regenerate (Joyce, 2003). The alteration of the integrity of the corneal endothelium might

cause corneal illnesses, for instance, Fuch's dystrophy, posterior polymorphous dystrophy or congenital hereditary endothelial dystrophy (Joyce, 2003).

2.3. Cell culture models

Corneal cell culture models are used for *in vitro* studies of different aspects of the ocular barrier that represents a major impairment for drug delivery and consequently for the development of pharmacological strategies in ophthalmology. These models have been extensively used to investigate the permeation, metabolism, toxicity and active transport of drugs and to predict ocular bioavailability. Cell culture models offer several advantages over animal testing for better controlling the experimental conditions and higher experimental reproducibility. Furthermore, cultured cells provide a constant and predictable source of research material for continuous experimental work. However, cell culture models are limited to simulate physiologically relevant characteristics including the precorneal drainage, blinking frequency as well as the lacrimal fluid composition that influence drug absorption, distribution, metabolism and elimination. Two types of cultures, primary cultures and immortalized corneal cell lines, are commonly used for establishing corneal cell models and are shortly described below (Kruszewski *et al.,* 1997; Hornof *et al.,* 2005, Reichl, 2008; Reichl and Becker, 2008).

2.3.1. Primary cell cultures

The cornea was identified as the principal barrier to ophthalmic drugs after topical application to the eye. Against this background, corneal epithelial cell cultures have been developed and served as a basis for the subsequent development of reconstructions of the complete human cornea. These corneal models have been developed as substitutes or supplements to animal models for toxicology and drug absorption studies (Castro-Muñozledo, 2008; Reichl *et al.,* 2011). Different explantation and culture techniques using tissues of human and animal origin have been improved with the aim of establishing a well-defined corneal model to be used for the above-mentioned purposes (Reichl *et al.,* 2011). However, human tissue is not readily available since healthy donor corneas are needed for keratoplasty (Lagali *et al.,* 2007; Niederer *et al.,* 2007; Hackett *et al.,* 2011). Consequently, primary animal corneal cells, mainly from rabbits (Kawazu *et al.,* 1998) cattle and pigs (Tegtmeyer *et al.,* 2001; Reichl and Müller-Goymann, 2001), are frequently used. Rabbit tissue proved to be particularly suitable for such studies since primary rabbit cells have a higher proliferative capacity than other species. They are readily available and data obtained with these models are highly comparable with data from *in vivo* studies as the majority of *in vivo* experiments are carried out with rabbits (Van Horn *et al.,* 1977; Reichl *et al.,* 2011). The expression of tight junctions in *in vitro* models is measured indirectly based on the transepithelial electrical resistance

(TEER) and the apparent permeability coefficient (P_{app}) of paracellular permeations markers (mannitol and sodium fluorescein). Both are parameters to evaluate the tightness of the epithelial barrier, which is an aspect that needs to be considered in drug absorption studies. In addition, cell culture conditions, such as cell growth media, coating materials, permeable supports or cultivation under Air-liquid interface (ALI) conditions, are important factors for the formation of tight junctions and the development of a multilayered corneal epithelium (Chang *et al.,* 2000). Therefore, primary rabbit corneal cell cultures solely established with epithelial cells cultured on permeable supports under liquid-covered conditions do not seem to be suitable for drug absorption studies because these models display relatively low TEER values of about 150 Ohm·cm² (Kawazu *et al.,* 1998; Kawazu *et al.,* 1999; Sakanaka *et al.,* 2006) compared to the TEER values reported for excised rabbit cornea (3000 to 8000 Ohm·cm^2) (Klyce and Wong*,* 1977). Dey *et al*. also described low TEER values of about 200 Ohm·cm² for a rabbit cell culture model (Dey *et al.,* 2003). However, it was noted that other primary cell cultures based on rabbit corneal epithelial cells with improved culture conditions (e.g. media supplements, ALI, seeding density), which have also been established for investigating the *in vitro* absorption of hydrophilic and lipophilic drugs, form a tight epithelial barrier of about 2300-5000 Ohm·cm^2 (Chang *et al.,* 2000; Scholz *et al.,* 2002; Becker *et al.,* 2005), which was similar to that of excised cornea. It has been described that primary corneal epithelial cell cultures with acceptable TEER values of about 400 Ohm·cm^2 (Reichl and Becker*,* 2008) are suitable for investigating the transport of hydrophilic and moderately lipophilic drugs but not that of highly lipophilic drugs (Reichl 2008, Hahne and Reichl*,* 2011). Within the scope of permeation studies, primary cell culture models, which also include a stromal layer achieved P_{app} values for highly lipophilic drugs, which were better comparable to excised cornea. These experiments underlined the importance to include both corneal cell layers, the epithelial and the stromal layer, in order to obtain barrier properties similar those of excised animal cornea to evaluate drugs with a wide range of different physicochemical properties (Tegtmeyer *et al.,* 2001; Reichl and Müller-Goymann*,* 2001; Reichl*,* 2008). Nevertheless, due to the low proliferative capacity and decreasing lifespan that primary cells display after several weeks in culture, the frequent isolation of cells is necessary. Thus, primary cell cultures are probably not the most adequate model to carry out larger scale screening test of new drugs and ophthalmic excipients or to evaluate novel ocular drug delivery systems (Toropainen *et al.,* 2001; Castro-Muñozledo*,* 2008). As a consequence, immortalized cell lines based on cells of human and animal origin were developed as alternatives to overcome the disadvantages of primary cell cultures (Castro-Muñozledo*,* 2008).

2.3.2. Immortalized corneal cells

In the last few years, several immortalized corneal epithelial cell lines have been established using cells from rabbits (Araki *et al.,* 1993), rats (Araki *et al.,* 1994) and humans (Kahn *et al.,* 1993; Araki-Sasaki *et al.,* 1995; Offord *et al.,* 1999). The particular advantage of immortalized cell lines is that they can be maintained in culture for a long time period without any change in their growth and morphological characteristics. Moreover, they can be stored in liquid nitrogen and used at any time for research purposes (Griffith *et al.,* 1999; Reichl and Becker, 2008; Toropainen *et al.,* 2001). However, only few of these cell lines have been used as an alternative model within the scope of *in vitro* drug absorption studies. The rabbit corneal epithelial cell line (RCE) is one example of these cell lines. A recombinant SV40-adenovirus vector was employed by Araki *et al*. to immortalize corneal epithelial cells from rabbits and rats (Araki *et al.,* 1993; Araki *et al.,* 1994). In culture, the RCE cell line displayed the cobblestone shape of corneal epithelial cells (Araki *et al.,* 1993), and has been used as *in vitro* model for drug absorption studies and for investigating the fluid transport through the epithelial cell layers (Burgalassi *et al.,* 2004; Yang *et al.,* 2000). However, this cell line did not seem to be an appropriate option for drug transport experiments as it was not able to form a tight epithelial barrier (TEER values of approximately 150 Ohm·cm²). Another rabbit corneal cell line, which is widely used is the Statens Seruminstitut rabbit cornea (SIRC) cell line. It was established by M. Volkert of the Seruminstitut, Copenhagen (Niederkorn *et al.,* 1990). In culture, these cells exhibit multiple elongated, epithelial-like cell layers but also a fibroblast phenotype (Niederkorn *et al.,* 1990; Hutak *et al.,* 1997). The SIRC cell line has been used for assessing toxicity and eye irritating effect of chemical substances, pharmaceutical excipients or ocular drugs (North-Root *et al.,* 1985; Ohno *et al.,* 1998; Tani *et al.,* 1999; Ayaki and Iwasawa, 2010; Ayaki *et al.,* 2011). This cell line has also been used for *in vitro* drug absorption studies. Goskonda *et al.* reported TEER values of about 2000 Ohm·cm² and permeability data similar to that obtained for isolated rabbit cornea (Goskonda *et al.,* 1999). In contrast, other groups obtained TEER values of only about 100 - 200 Ohm·cm² and were thus not able to achieve a tight barrier. As a consequence of this, high P_{app} values were obtained for hydrophilic and lipophilic permeation markers compared to those obtained with isolated rabbit cornea (Tak *et al.,* 2001; Burgalassi *et al.,* 2004; Sakanaka *et al.,* 2006). Thus, the SIRC model is not suitable for drug absorption studies.

The most commonly used human corneal epithelial cell line (HCE-T) was established by Araki-Sasakai *et al*. in 1995. A SV40-simian adenovirus without the origin of SV40 viral replication was employed to transfect human corneal epithelial cells obtained from a 49-year-old woman with maxillary sinus carcinoma (Araki-Sasaki *et al.,* 1995). In culture, HCE-T cells possess characteristics similar to those of human corneal epithelial cells. They exhibit the

cobblestone-like shape and develop desmosomes and microvilli, which are typical morphological features of human corneal epithelial cells (Araki-Sasaki *et al.,* 1995). Toropainen *et al*. was the first investigation group, which used these cells to establish an *in vitro* model of the corneal epithelium for drug absorption studies. HCE-T cells cultivated on collagen/laminin coated permeable filters under ALI conditions showed TEER values ranging from about 400 - 800 Ohm·cm². The cultivated *in vitro* corneal epithelium formed about seven cell layers, which were comparable to the stratified human corneal epithelium. Permeability assays conducted with hydrophilic and lipophilic permeation markers demonstrated the capacity of the *in vitro* HCE-T corneal model to distinguish between different physicochemical properties of the used substances (Toropainen *et al.,* 2001). For instance, the HCE-T culture model has been used, to understand how drugs permeate through the corneal epithelium and to evaluate the corneal absorption and desorption rate constant after the topical administration of ocular drugs (Toropainen *et al.,* 2003; Ranta *et al.,* 2003). Other groups used HCE-T cells in drug absorption studies in order to investigate the influence of formulation parameters on the permeability of the *in vitro* corneal epithelium and, furthermore, to perform comparative investigations with other *in vitro* corneal cell models. Within these investigations, the TEER values reported ranged from about 330 - 600 Ohm·cm² (Becker *et al.,* 2007; Becker *et al.,* 2008). Other investigations group noted TEER values, which were at about 400 Ohm·cm² (Nagai *et al.,* 2008), about 200 Ohm·cm² (Kimura *et al.,* 2009; Yanai *et al.,* 2009) or approximately 1200 Ohm·cm² (Reichl*,* 2008).

Another human corneal cell line is the CEPI-17-CL 4 cell line, which was developed by Offord *et al*. (Offord *et al.,* 1999). In culture, CEPI-17-CL 4 cells have the cobblestone shape of human corneal epithelial cells. Several investigation groups have used the CEPI-17-CL 4 cell line to study the human corneal epithelium in vitro and also for drug absorption studies. However, CEPI-17-CL 4 based *in vitro* corneal models showed low TEER values of about 100 Ohm·cm² due to, which this cell line is considered to be less suitable for drug absorption studies (Crider *et al.,* 2003; Reichl *et al.,* 2004; Reichl *et al.,* 2005; Reichl*,* 2008).

In addition to the mentioned cell lines, human corneal epithelial cell models (established using immortalized cell lines) are also commercially available. Examples of these models are the model created by Skinethic laboratories (sHCE model), the Epiocular® model by MatTek Corporation and the Clonetics® model (cHCE) by Lonza (Reichl *et al.,* 2011). They have been used for evaluating the irritation potential of ophthalmic excipients, cosmetics and chemical substances to the ocular surface and, furthermore, they have been applied in validation studies with the aim of replacing the Draize eye test (Cotovio *et al.,* 2007; Doucet *et al.,* 2006; Van Goethem *et al.,* 2006; Khoh-Reiter and Jessen*,* 2009; Cotovio *et al.,* 2010; Jones *et al.,* 2001; Barile*,* 2010).

With regard to the tightness of the epithelial barrier of these models to be used for drug absorption studies, the sHCE and Epiocular® model showed TEER values of about 100 - 200 Ohm·cm², which are far below the TEER values obtained in experiments with *ex vivo* corneal epithelia (Becker *et al.,* 2008; Reichl*,* 2008). Consequently, the permeability of both models to the hydrophilic permeation markers mannitol and sodium fluorescein was about 10 to 60 times higher than that of excised cornea (human, rabbit and bovine cornea) (Becker *et al.,* 2008; Reichl*,* 2008). For these reasons, these *in vitro* corneal models are not suitable for drug absorption studies. In contrast, it has been observed that the cHCE model is able to form a tight barrier similar to that of the corneal epithelium of excised human cornea (Becker *et al.,* 2008). The cHCE model showed TEER values of about 800 Ohm·cm² and the permeability to sodium fluorescein comparable to those observed for excised human and rabbit cornea (Becker *et al.,* 2008). Other drug absorption studies conducted with 11 drugs have indicated that the permeability characteristic of the cHCE model is similar to that of excised rabbit cornea (Xiang *et al.,* 2009).

A short summary of the primary epithelial cells and immortalized cell lines used to establish *in vitro* corneal cell models for drug absorption studies is provided in the Table 2-1.

Table 2-1: Summary of primary epithelial cells and immortalized cell lines for constructing *in vitro* corneal cell models

Model	Origin	Characteristics	References
Primary rabbit corneal epithelium	(1) Established by Kawazu *et al.,*1998); (2) Established by Chang *et al.,* 2000)	Rabbit origin; low TEER values of ≈150 Ohm·cm² for the model of Kawazu *et al*. and TEER values > 2000 Ohm·cm² for the model of Chang *et al*.; used for drug permeation studies	Kamazu *et al.,* 1998, 1999, 2006; Chang *et al.,* 2000; Dey *et al.,* 2003; Scholz *et al.,* 2002; Becker *et al.,* 2005; Sakanaka *et al.,* 2006
RCE	Established by Araki *et a*l.,1993)	Immortalized cell line based on albino rabbit cornea by viral transduction (SV40); low TEER values of ≈ 150 Ohm·cm²; used to study drug permeation	Yang *et al.,* 2000; Burgalassi *et al.,* 2004
SIRC	Established by M. Volkert of Seruminstitut Copenhagen (Niederkorn *et al.,* 1990)	Immortalized cell line based on rabbit cornea; low TEER values of ≈100 - 200 Ohm·cm²; fibroblast phenotype; used for permeability, transporter, esterase activity and toxicity studies	Niederkorn *et al.,* 1990; Hutak *et al.,* 1997; Tak *et al.,* 2001; Dey *et al.,* 2003; Majumdar *et al.,* 2003; Becker *et al.,* 2008; Goskonda *et al.,* 1999; 2000; North-Root *et al.,* 1985; Ohno *et al.,* 1998; Tani *et al.,* 1999; Ayaki and Iwasawa*,* 2010; Ayaki *et al.,* 2011

HCE-T	Established by Araki-Sasaki *et al*., 1995)	Immortalized human cell line developed by viral transduction (SV40); tight multilayered epithelium with TEER values of 400 - 800 Ohm·cm² for the model of Toropainen *et al*.; TEER values of 330 - 600 Ohm·cm² for the model of Becker *et al.* TEER values of 1200 Ohm·cm² for the model of Reichl. and 200 Ohm·cm² for the model of Kimura *et al*.; used to study drug transport, transporter expression	Toropainen *et al.,* 2001, 2003; Ranta *et al.,*2003; Becker *et al.,* 2007, 2008; Reichl*,* 2008; Nagai *et al.,* 2008; Kimura *et al.,* 2009; Yanai *et al.,* 2009
CEPI-17-CL 4	Established by Offord *et al.,* 1999)	Immortalized human cell line developed by viral transduction (SV40); low TEER values of 100 Ohm·cm² for the model of Reichl.; used for drug transport and toxicity experiments	Offord *et al.,* 1999; Crider *et al.,* 2003; Reichl *et al.,* 2004; Reichl *et al.,* 2005; Reichl*,* 2008; Reichl and Becker*,* 2008
sHCE	Established at SkinEthic Laboratories	Immortalized human corneal epithelial cells; low TEER values of ≈ 100 - 200 Ohm·cm²; used for drug transport and toxicity studies and *in vitro* experiments on ocular irritancy	Nguyen *et al.,* 2003; Cotovio *et al.,* 2007, 2010; Doucet *et al.,* 2006; Van Goethem *et al.,* 2006; Becker *et al.,* 2008; Reichl*,* 2008
Epiocular®	Established at MatTek Corporation	Non-corneal origin (human derived epidermal ketinocytes); multilayered epithelium; low TEER values of ≈ 100 Ohm·cm²; used for drug transport and toxicity experiments	Jones *et al.,* 2001; Reichl*,* 2008; Barile*,* 2010
cHCE	Established at Cambrex Bio Science in 2006	Immortalized human cell line developed by viral transduction (HPV-16 ED/E7); tight multilayered epithelium with TEER values of ≈ 800 Ohm·cm²; used for drug transport studies	Becker *et al.,* 2008

RCE: Rabbit corneal epithelial cell line; SIRC: Statens Seruminstitut rabbit corneal epithelial cell line; HCE: Human corneal epithelial cell line, SV-40: Simian adenovirus; HPV: Human papilloma virus

2.3.3. Organotypic cell culture models

When designing organotypic cell culture models, the main objective is to reconstruct corneal tissue, which possesses the same characteristics and mimics the functions of its *in vivo* equivalent. Such a model can be used to investigate, for example, the toxicity of pharmaceutical excipients and active drugs, the drug transport, the cell proliferation and the influence of formulation parameters. Furthermore, it can also be employed to develop and improve drug delivery systems. These models are prepared using primary or immortalized corneal cells (Reichl and Becker*,* 2008; Hornof *et al.,* 2005; Barar *et al.,* 2009). The reconstruction of the corneal barrier with primary corneal cells is a demanding task since the cells must be isolated from the animal or human tissue in order to be used to reconstruct layer by layer the corneal barrier (Minami *et al.,* 1993; Germain *et al.,* 1999). For this purpose, the culture conditions, such as the co-cultivation of the epithelial cells with only keratocytes or with both keratocytes and endothelial cells as well as the ALI conditions, are important for obtaining a well-defined multilayered corneal epithelium, a high expression of

tight junctions and TEER values similar to those achieved with *in vivo* tissue (Friend *et al.,* 1982; Chan and Hasche*,* 1983; Orwin and Hubel*,* 2000; Chang *et al.,* 2000; Toropainen *et al.,* 2001). For *in vitro* drug absorption studies, *in vitro* corneal models established only with epithelial cells, which achieve sufficiently high TEER values are suitable to evaluate the permeation of hydrophilic and moderately lipophilic drugs but not highly lipophilic drugs (Becker *et al.,* 2008; Reichl*,* 2008). In contrast, *in vitro* corneal models co-cultivated with stromal keratocytes in stromal biomatrix can be used to investigate the transport of highly lipophilic drugs as their permeability characteristics better match those of *in vivo* tissue (Reichl and Müller-Goymann*,* 2001; Reichl and Müller-Goymann*,* 2003; Tegtmeyer *et al.,* 2004; Reichl*,* 2008).

Nevertheless, there are some problems related to the use of primary cells to construct *in vitro* corneal models. For example, these problems include that the cells must frequently be isolated, no predefined standard protocols to establish the cornea models are available, which leads to a poor reproducibility of results, the transfer of these protocols between laboratories is difficult and the transferability of results obtained from animal-based models to humans is questionable (Hahne and Reichl*,* 2011). Immortalized human corneal cells have been used to overcome the above-mentioned issues (Griffith *et al.,* 1999). Some advantages of immortalized cell lines have already been mentioned in section 2.3.2. Furthermore, the protocols to establish *in vitro* corneal models using the above cells (i.e. HCE-T cell line) are easier to standardize than those of primary cell cultures, which has been confirmed by the reduction in the intralaboratory variability of permeation results (Hahne and Reichl*,* 2011).

2.3.4. Three-dimensional corneal models

The corneal models are classified according to their culture complexity: a monolayer epithelium, a stratified epithelium, the co-culturing of epithelial and stromal layers and cornea equivalent systems. Within this classification, the co-culture and cornea equivalent models are considered to be three-dimensional (3D) corneal models (Huhtala *et al.,* 2008).

In vitro 3D corneal models are frequently reconstructed layer by layer: First, endothelial cells, keratocytes and epithelial cells are isolated from the corneal tissue. Second, cells are embedded in a matrix comparable to the *in vivo* conditions. Third, the corneal cells are co-cultured to reconstruct the whole corneal barrier and are exposed to the ALI conditions to promote the formation of a multilayered corneal epithelium (Minami *et al.,* 1993). Over time, the improvement of the cell culture conditions has contributed to the understanding of the role of corneal structures (e.g. the corneal endothelium), which promote the differentiation of the epithelial cells and the formation of the basement membrane. The expression of hemidesmosomes, anchoring fibrils and the cell-cell interaction might lead to the differentiation of the epithelial cells as well as the synthesis and assembly of basement

membrane components (Zieske *et al.,* 1994; Schneider *et al.,* 1999). Human corneal models have been reported to be able to produce α and β integrin subunits, which have been closely linked to the wound healing process after corneal damages, as well as laminin type VII and collagen (Germain *et al.,* 1999; Germain *et al.,* 2000). All these aforementioned (3D) corneal models were basically developed, firstly, to improve the cell culture techniques used to reconstruct *in vitro* corneal cell models, which are equivalent to their *in vivo* counterparts, secondly, to evaluate the eye irritation potential of chemicals in order to replace the Draize test and, thirdly, to demonstrate that the *in vitro* reconstruction of the human cornea can possess morphological characteristics and basement membrane components similar to its *in vivo* equivalent. However, the suitability of these (3D) corneal models for *in vitro* drug absorption studies has not yet been evaluated. Tegtmeyer *et al*. developed an *in vitro* corneal model exclusively from bovine primary cells, and this (3D) corneal model was the first to be used for drug absorption studies. The morphology of the corneal epithelium of this model was similar to that of the epithelium of excised bovine cornea and furthermore achieved similar results with regard to the permeability rates for drugs (pilocarpine hydrochloride and timolol maleate) in different ophthalmic formulations (Tegtmeyer *et al.,* 2001; Tegtmeyer *et al.,* 2004). Similar results have been reported for a (3D) corneal model based on porcine primary cells (Reichl and Müller-Goymann, 2001; Reichl and Müller-Goymann, 2003). In addition, Reichl *et al*. established a (3D) human corneal model, which consists of immortalized human endothelial cells (Bednarz *et al.,* 2000), primary cultured human keratocytes and the immortalized human epithelial cell line CEPI-17-CL4 (Offord *et al.,* 1999), which is also described in section 2.3.2. The morphology of the epithelium of this reconstructed (3D) human corneal model is similar to that of an *in vivo* epithelium (Reichl *et al.,* 2004). This reconstructed human corneal model has been used in several drug absorption studies carried out with different ophthalmic formulations. With regard to pilocarpine hydrochloride, befunolol hydrochloride, and hydrocortisone in aqueous formulations, the model´s permeability to these substances was almost similar to that of isolated porcine cornea (Reichl *et al.,* 2004). Other drug absorption studies demonstrated that the permeability of this model was similar to that observed for excised human cornea with regard to six different ophthalmic drugs (Reichl *et al.,* 2005). However, *in vitro* corneal models based on CEPI-17-CL4 cells achieved similar P_{app} values for mannitol (highly hydrophilic permeation marker) and timolol maleate (moderately lipophilic drug) due to, which these models exhibited low TEER values (< 100 Ohm·cm²). For this reason, (3D) human corneal model reconstructed with CEPI-17-CL4 cells are not suitable to investigate the transcorneal permeation of drugs with widely differing physicochemical properties (Reichl, 2008). In the same study, Reichl demonstrated that the corneal models based on the aforementioned HCE-T cell line (Araki-Sasaki *et al.,* 1995) show barrier characteristics

similar to those of isolated animal cornea and thus these corneal constructs are able to differentiate between hydrophilic and lipophilic substances. In further studies, Reichl and co-workers established a human Hemicornea (HC) construct (stroma-epithelium) using immortalized human keratocytes (Zorn-Kruppa *et al.,* 2005), which were embedded in a collagen matrix (stromal equivalent) and covered with the multilayer corneal epithelium (HCE-T cells). At first, this HC construct was cultivated in a serum-free growth medium under liquid covered conditions and then under ALI conditions. The epithelium of the HC construct developed a morphology and tight epithelial cell layers, which were comparable to those of *in vivo* human cornea, as is illustrated in Figure 2-3 (Hahne and Reichl*,* 2011). With regard to sodium fluorescein, FD-4 and rhodamine B, the performed *in vitro* absorption studies indicated that the HC construct possesses permeability properties, which lie between those of excised rabbit and porcine cornea. The anatomy of porcine corneal tissue rather resembles that of the human cornea than that of rabbit corneal tissue. This led to the assumption that the permeability characteristics of the HC construct are similar to those of the human cornea. Consequently, the permeability values obtained for the aforementioned substances indicate that the actual permeability of the human cornea lies between the permeability of rabbit and porcine cornea. In addition, the reproducibility of the P_{app} values obtained with the above substances for the HC construct were significantly higher than those obtained for isolated rabbit and porcine cornea (Hahne and Reichl*,* 2011). However, additional investigations need to be carried out before the HC construct can be considered as an alternative to animal experimentation in preclinical studies. These investigations include the prevalidation of the model in order to demonstrate the interlaboratory reproducibility of data, permeability assays with a high number of substances covering a broad range of different molecular attributes for the purpose of evaluating the capacity of the HC construct to differentiate between hydrophilic and moderately and highly lipophilic drugs, the comparison of the permeability of the HC construct to that of human cornea, as well as the evaluation of the influence of formulation parameters (buffer solutions at different pH levels, the osmolality and ophthalmic adjuvants, such as EDTA, calcium and preservatives) on the permeability of the HC construct. Against this background, the investigations conducted within the scope of this thesis were based on the HC construct established by Reichl and co-workers.

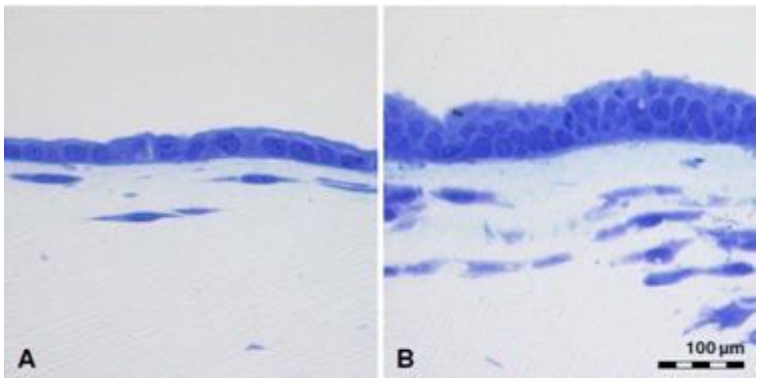

Figure 2-3: **A.** Cross section and toluidine blue staining of the HC construct. Monolayer of HCE-T cells on the stromal matrix containing HCK cells after one week of cultivation under liquid-covered conditions. **B.** Formation of a stratified corneal epithelium after 3 further days of cultivation under ALI conditions similar to the human cornea. Scale bar = 100 µm (Hahne *et al.,* 2012)

2.4. Ocular drug absorption

A high number of ocular diseases, such as infections and disorders (e.g. glaucoma or dry eye syndrome), are mostly treated by means of topical administration of ophthalmic solutions, which are the predominant pharmaceutical form available on the market (Bourlais *et al.,* 1998, Urtti*,* 2006). Following the instillation of the medication, ocular drug absorption can occur through the corneal or non-corneal route.

2.4.1. Corneal absorption route

The ocular bioavailability of topically applied drugs is very poor because the cornea forms a tight barrier and the drug is rapidly removed from the corneal surface (lacrimal drainage) (Järvinen and Järvinen*,* 1996; Urtti*,* 2006). The permeation of the drug through the corneal barrier takes place by means of passive diffusion and/or an active transport. The latter is dependent on the expression level of the transport proteins (Mannermaa *et al.,* 2006). A high proportion of drugs undergo passive diffusion through the corneal epithelium, which involves the transport via the paracellular and transcellular pathway. The epithelium is the main corneal barrier for hydrophilic drugs (Lee*,* 1990). In contrast, lipophilic drugs take the transcellular pathway to penetrate the corneal epithelium. However, their transport is affected by the hydrophilic characteristics of the corneal stroma, which constitutes 90% of the cornea (Schoenwald and Huang*,* 1983; Huang *et al.,* 1983). The corneal endothelium is a monolayer of cells with a thickness of 5 µm (Whikehart*,* 2010) and is considered to be a permeable layer that contributes only minor to the corneal barrier. Thus, it can be assumed that the corneal endothelium has larger intercellular gaps than the corneal epithelium (Huang *et al.,* 1983). Beside the morphological characteristics of the cornea, the level of permeation of drugs is also influenced by their physicochemical properties, such as the solubility, molecular weight, charge and the partition coefficient, among others (Grass and Robinson*,* 1988; Liaw and

Robinson, 1992; Huang *et al.,* 1989; Liaw *et al.,* 1992; Rojanasakul *et al.,* 1992; Sieg and Robinson, 1977; Brechue and Maren, 1993). Regardless of the tightness of the corneal epithelial cell layers, the transcorneal drug permeation is the predominant route, which drugs take in order to reach the aqueous humor (Urtti, 2006).

2.4.2. Non-corneal absorption route

The non-corneal absorption route includes the ocular tissue of conjunctiva and sclera, which are known as the conjunctival-scleral route. In contrast to the corneal route, large and hydrophilic drugs, e.g. peptide drugs, are mainly absorbed via the conjunctival-scleral route (Hornof *et al.,* 2005; Urtti, 2006). The conjunctiva facilitates the transport of topically applied drugs into the blood stream but not into the intraocular tissue as it is vascularized and has a large surface. The conjunctiva is a fine mucous membrane, which covers approximately the area ranging from the anterior part of the sclera to the inner part of the eyelids, not including the cornea. The conjunctiva is formed by up to 15 layers of epithelial cells, which make it a multilayered epithelium (Andrés-Guerrero and Herrero-Vanrell, 2008; Bourlais *et al.,* 1998). Nevertheless, its permeability with regard to non-lipophilic substances, even those with high molecular weight, is significantly higher than that of the cornea. As a consequence, the conjunctiva is considered a leaky tissue (Huang *et al.,* 1989; Hämäläinen *et al.,* 1997). The intercellular gap junctions, the pore density and the turnover of lacrimal fluid of the conjunctiva are larger than those of the cornea due to, which the drug is rapidly eliminated (Hämäläinen *et al.,* 1997). The sclera is a robust fibrous connective tissue formed by collagen type I, possessing protective properties (Watson and Young, 2004; Rada *et al.,* 2006). In terms of permeability, the sclera has a higher permeability to drugs than the conjunctiva and cornea since the molecular size of drugs has less influence on the absorption through the sclera than through the cornea (Hämäläinen *et al.,* 1997). This absorption route is still being investigated in order to improve the ophthalmic drug delivery to intraocular structures (the aqueous humor and retina).

Despite the differences in the permeability of the cornea and conjunctiva-sclera, the majority of drugs that are topically administered into the eye are transported via the corneal route (Urtti, 2006).

2.4.3. Formulation parameters

2.4.3.1 pH

The eye can tolerate ophthalmic preparations at pH values ranging from 3.5 to 9.0 (Gibson, 2009). However, in order to ensure maximum comfort, ophthalmic preparations should have a pH value of about 7.4, which corresponds to the pH of lacrimal fluid (Gibson, 2009).

In ophthalmic preparations, the buffering capacity and the pH of ophthalmic vehicles should be able to maintain the stability of the active compound. For this reason, buffer solutions are used to avoid changes in the pH values of ophthalmic preparations (Mitra and Mikkelson, 1987; USP 29, 2006).

The stability of the active compound in ophthalmic formulations needs to be conserved to ensure that its potential effect is maintained. As a consequence, stability studies aim to demonstrate the effectiveness of the active compound (Cha *et al.,* 2001). The stability and delivery form of active compounds are two of the most relevant parameters in the development of formulations (Attwood and Poust, 2009). For instance, buffering agents with a buffering capacity not exceeding 0.05% and at pH of between 5 and 6 were employed by Akram *et al.* to ensure the stability of 1.0% prednisolone acetate formulations (Akram *et al.,* 2010). Apparently, chemical reactions can lead to an increase in the formation of degradation products and thereby to a further decrease in the amount of the active substance in the pharmaceutical form. As a consequence, there is a loss in the effectiveness of the active compound and an increase in adverse reactions. Therefore, a degradation of active compounds should be avoided. Investigations have revealed that degradation products often produce more unwanted side effects than the active compound (Attwood and Poust, 2009). Substantial modifications in the physical appearance of the pharmaceutical preparation, such as a mottling of tablets, disruption of emulsions or caking of the suspension are also taken into account (Attwood and Poust, 2009). A non-complex drug molecule is indefinitely stable in aqueous solution. Accordingly, the stability of pharmaceutical preparations must be guaranteed in order to ensure that more than 90% of the labeled concentration is maintained under storage conditions. It has been shown that ophthalmic drugs in aqueous solutions are in some cases more stable in acid environments than in neutral or alkaline environments; particularly when the molecular form is a weak base. In order to avoid the rapid formation of undesirable by-products, the protonation of the active compounds is achieved by maintaining the molecular form at an acid pH. Moreover, the corneal surface, the pH and the buffer capacity of the tear fluid are factors that must be considered during the development or improvement of ophthalmic preparations (Fiscella, 2008).

Most drugs are either weak acids or weak bases and they exist in ionized and unionized forms in aqueous solutions. The pKa indicates the ionizability of a compound. A molecule might lose or gain a proton when it is placed into an aqueous solution. Whether a molecule is protonated or deprotonated depends on its pKa and on the pH of the buffer solution. Therefore, the pKa is defined as the negative logarithm of the acid-ionization constant. The term pKa can be employed for both acids and bases (Kerns and Di, 2008).

The lipophilicity of molecular markers and drugs is determined either by measuring the octanol-water partition coefficient, which is usually expressed as log *P* (see Equation 2-1), or

by measuring the so-called log *D*, which is the distribution coefficient of all forms of a molecule (non-ionized and ionized solute between octanol and water). The log *D* of ionizable molecules of molecular markers and drugs is pH-dependent, and is calculated as is shown in Equation 2-2.

Equation 2-1:

$$P = \frac{[non-ionized\ solute]_{octanol}}{[non-ionized\ solute]_{water}}$$

Equation 2-2:

$$D = \frac{[non-ionized\ solute + ionized\ solute]_{octanol}}{[non-ionized\ solute + ionized\ solute]_{water}}$$

A sigmoidal relationship was observed between the lipophilicity (i.e. log *P*) and the P_{app} values of some β-blockers (see Figure 2-4). Thus, the drug lipophilicity influences the passive drug diffusion through the corneal barrier (Toropainen *et al.,* 2003). In addition, buffer solutions and pH may affect the lipophilicity of a drug and consequently its permeation through the corneal barrier (Schoenwald and Huang, 1983; Wang *et al.,* 1991; Suhonen *et al.,* 1991; Suhonen *et al.,* 1998).

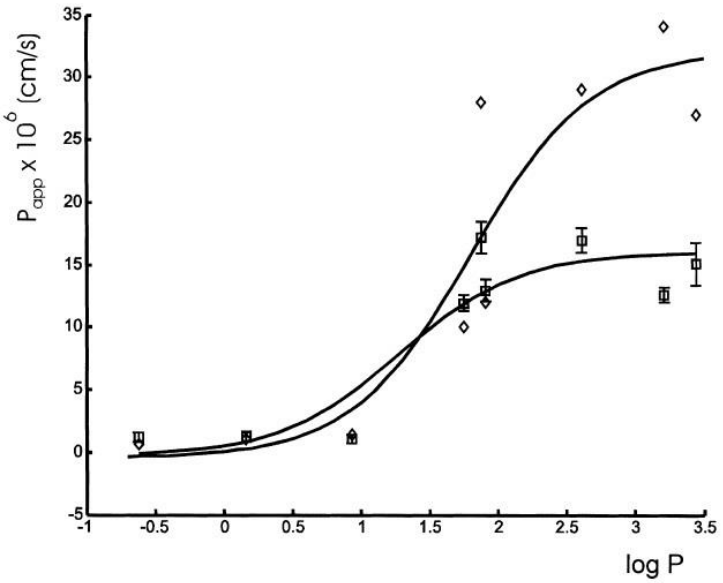

Figure 2-4: Influence of drug lipophilicity expressed as log *P* on the permeability of the HCE-T model (squares) expressed as permeability coefficient and on isolated rabbit cornea (diamonds) with regard to eight β-blockers (Toropainen *et al.,* 2003)

2.4.3.2 Osmolality

The tonicity is important with regard to pharmaceutical preparations, which are instilled into the eye, since it may negatively influence the bioavailability of drugs due to excessive lacrimation (Conrad *et al.,* 1978). The lacrimal fluid has a tonicity value of approximately 279 mOsmol·kg^{-1}, which corresponds to that of 0.9% NaCl solution. In theory, ophthalmic solutions should possess this tonicity level. The eye can tolerate a wide range of tonicity values (from 0.5% to 1.5% NaCl equivalents) without producing any irritancy effect. Notwithstanding, some ophthalmic solutions are outside of this range (Gibson*,* 2009).

2.4.3.3 EDTA and calcium

The tight junctions (TJs) of the corneal epithelium (see Figure 2-2) limit the penetration of substances and solutes through the corneal epithelium (Ban *et al.,* 2003a). Together with transmembrane proteins (claudin and occludin), membrane-associated proteins (ZO-1, ZO-2 and ZO-3), adherence junctions and desmosomes, the TJs form the so-called “junctional complex” (Furuse *et al.,* 1994; Ban *et al.,* 2003a; Cereijido *et al.,* 2004). TJs are laterally connected to each other, building strands in order to seal the intercellular spaces (Furuse *et al.,* 2001) and are located on the surface of the human corneal epithelium. ZO-1 was found in the most apical cells of the epithelium and between the basal and wing cells. Occludin was only found in the lateral connections of superficial cells (cell-cell borders) but not in the apical membrane (Anderson *et al.,* 1993; Ban *et al.,* 2003a). Other kinds of associated proteins, such as actin, myosin and α-catenin, were found under the apical junctional complex. The modulation of the myosin contraction and movement is, among others, regulated by the entering of extracellular calcium into cells. This process occurs in the presence of ATP (adenosine triphosphate) and phosphorylation of the calcium/calmodulin-dependent protein kinases, which lead to the opening of TJs. As a consequence, the paracellular permeability is promoted (Salama *et al.,* 2006). Actin cytoskeleton plays an important role in the regulation of calcium storage, release and store-coupled calcium entry (Lange and Gartzke*,* 2006).

Excipients such as ethylenediaminetetraacetic acid (EDTA) are often used in ocular drug formulations in order to improve the antimicrobial effect of preservatives. EDTA is frequently combined with benzalkonium chloride (preservative agent) with the aim to increase the antibacterial activity against strains of *Pseudomonas* (Furrer *et al.,* 2002; Heydari *et al.,* 2013). Moreover, EDTA is a chelator of heavy metals and able to lower the concentration of free calcium ions in aqueous solutions (Morrison and Khutoryanskiy*,* 2014). Therefore, *in vivo* permeation studies conducted in rabbits showed that EDTA increased the corneal permeability to hydrophilic and lipophilic substances by influencing the TJ function (Grass *et al.,* 1985). Other *in vitro* permeation studies carried out with isolated rabbit cornea have

suggested that EDTA causes a disruption of the TJs, which is reflected in the depletion of calcium from the ocular surface resulting in an increase in the corneal permeability to substances (Rojanasakul and Robinson, 1991). Moreover, similar results were obtained with other *in vitro* cell culture models using oxyntic cells of gastric glands, pancreatic acinar cells or Caco-2 cells (Contreras *et al.,* 1992; Ma *et al.,* 2000). This evidence supports the assumption that EDTA and calcium may also affect the *in vitro* corneal permeability of the HC construct.

2.4.4. Preservatives

In the 1960s, there were incidents of eye ointments contaminated with Pseudomonas aeruginosa, as a result of, which preservatives have been used in ophthalmic medication thenceforth (Furrer *et al.,* 2002). According to USP 29, multi dose packaging of ophthalmic preparations must contain a suitable substance or a mixture of preservatives, which prevents potential contamination and ensures its sterile condition after opening (USP 29, 2006). Thus, the main purpose of preservatives in ophthalmic preparations, artificial tears or contact lens solutions is to avoid the growth of microorganisms (Furrer *et al.,* 2002). During the development of new ophthalmic preparations or their improvement, it is of great importance to investigate the influence of preservatives on the transport of active compounds through the cornea as well as their ocular toxicity.

Different studies have demonstrated that the use of preservatives might alter the corneal permeability to drugs since a disruption of the corneal barrier was noted (Pfister and Burstein, 1976; Camber and Edman, 1987). Moreover, different kinds of preservatives are not only used in ophthalmic preparations but also nasal drops and some inhalers. It is still questionable whether the use of preservatives has adverse effects on the corneal and nasal epithelium is still questionable (Baudouin *et al.,* 2010; Marple *et al.,* 2004; Mallants *et al.,* 2007). Preservatives used at high concentrations can perhaps produce eye irritation or damage the ocular surface (Baudouin, 2008; Ayaki *et al.,* 2010). The following table shows the preservatives and the respective concentrations, which have been used in pharmaceutical preparations (Furrer *et al.,* 2002).

Table 2-2: Overview of ophthalmic preservatives (adapted from Furrer *et al.,* 2002)

Classification	Preservatives	Concentration [%(m/v)]	Optimal pH
Quaternary amoniums	Benzalkonium chloride (BAC)	0.004 - 0.02	---
	Cetrimide (Ce)	0.005	7 - 9
	Cetylpyridinium chloride	0.025	---
	Benzododecinium bromide	0.012	---
	Benzethonium chloride	0.01 - 0.02	---

Classification	Preservatives	Concentration [%(m/v)]	Optimal pH
	Polyquaternium-1 (Polyquad® – PQ-1)	0.001*	---
Mercuriales	Phenylmercuric nitrate/acetate/borate	0.002 - 0.004	4 - 10
	Thiomersal (Thio)	0.001 - 0.02	7 - 8
Alcoholes	Chlorobutanol	0.5	< 5.5
	Benzyl alcohol	0.5	< 5
	Phenoxyethanol	1.0	< 6
	Phenylethyl alcohol	0.5	< 5
Carboxylic acids	Sorbic acid	0.2	4.5
Phenoles	Methylparaben (MP)-propylparaben	0.1	4 - 9
Amidines	Chlorhexidine digluconate	0.005 - 0.01	5 - 8
Miscellaneous	EDTA	0.01 - 0.1	4 - 10
Stabilized oxychloro complex	Purite® (95.5% $NaClO_2$ – 0.05% $NaClO_3$)	0.005 – 0.0075**	---
"Self-preserved"	sofZia®	(see section 3.1.5)***	7.9

*(Paimela *et al.,* 2012); **(Rote-Liste, 2013; Kaur *et al.,* 2009); ***(Chowhan *et al.,* 2010)

To evaluate the effect of excipients commonly used in ophthalmic formulations on the *in vitro* corneal model HC, firstly the excipients of 28 frequently prescribed ophthalmic drugs listed in "Rote-Liste" (Drug information for Germany) were reviewed. As a result, it was noted that two ophthalmic formulations contain citrate, three borate, ten phosphate and three contain citrate and phosphate buffers, as well as ten are unbuffered ophthalmic preparations. With regard to the preservative used, it was found that ten of them contain BAC, three BAC and EDTA, four Ce and EDTA, one Thio, four Purite, two contain Polyquad® and four are preservative-free ophthalmic preparations. Currently, in Germany, there are apparently no pharmaceutical preparations available, which contain the preservative system sofZia®.

3. Materials and Methods

3.1. Materials

3.1.1. Immortalized human cell lines

The Table 3-1 presents the cell lines used in this study.

Table 3-1: Cell lines used to construct the human Hemicornea model

Cell line	Description	Supplier
HCE-T (Araki-Sasaki *et al.,* 1995)	Transfected by recombinant SV 40-adenovirus vector, human corneal epithelial cells	TUBS (Riken Bioresource Center, Koyadai, Tsukuba, Ibaraki,Japan, Catalog number: RCB 1384)
HCK (Zorn-Kruppa *et al.,* 2005)	Transfected by recombinant SV 40-adenovirus vector, human corneal keratocytes	TUBS (Dr. M. Zorn-Kruppa, Academy for Animal Protection - Deutscher Tierschutzbund e.V.

3.1.2. Permeation markers and substances

3.1.2.1 Permeation markers for the prevalidation of the HC construct

The Table 3-2 shows physicochemical properties and concentrations of permeation markers used in this study.

Table 3-2: Permeation markers used for permeability studies with the HC construct in KRB at pH 7.4

Substance	Chemical formula	Molecular weight [g·mol^{-1}]	*Log *P*	Concentration	Supplier
Sodium fluorescein (Na-FLU)	$C_{20}H_{10}Na_2O_5$	376.27	-1.52	10 µg·mL^{-1}	Sigma-Aldrich, Steinheim, Germany
Fluorescein isothiocyanate-dextran (FD-4)	$C_{21}H_{11}NO_5S$-$H(C_6H_{10}O_5)_xOH$	4400	-2.0	2500 µg·mL^{-1}	
Rhodamine B	$C_{28}H_{31}ClN_2O_3$	479.02	1.78	50 µg·mL^{-1}	

*Obtained from http://www.chemicalize.org (accessed at 12.07.2016), except for Na-FLU and FD-4 (Sakai *et al.,* 1997)

3.1.2.2 Ophthalmic drugs for the prevalidation of the HC construct

Some molecular properties of ophthalmic active compounds are depicted in Table 3-3. The ophthalmic active compounds were used for the prevalidation of the HC construct as well as for other drug permeability studies.

Table 3-3: Relevant ophthalmic drugs used to prevalidate the HC construct in KRB at pH 7.4

Substance	Chemical formula	Molecular weight [g·mol^{-1}]	*Log *P*	Concentration -Activity	Supplier
^{3}H-aciclovir	$C_8H_{11}N_5O_3$	225.20	-1.03	1.0 µCi·mL^{-1}	American Radiolabeled Chemicals, St. Louis, USA
^{3}H-bimatoprost	$C_{25}H_{37}NO_4$	415.57	2.63		
^{3}H-dexamethasone	$C_{22}H_{29}FO_5$	392.46	1.68		
Timolol maleate	$C_{13}H_{24}N_4O_3S$	316.42	1.34	75.0 µg·mL^{-1}	Biotrend, Cologne, Germany

*Obtained from http://www.chemicalize.org (data accessed on 12.07.2016)

3.1.2.3 Other relevant substances

The substances listed in Table 3-4 were used to evaluate the permeability properties of the HC construct.

Table 3-4: Summary of substances dissolved in KRB at pH 7.4 for *in vitro* ocular drug absorption

Substance	Chemical formula	Molecular weight [g·mol^{-1}]	*Log *P*	Concentration -Activity	Supplier
^{3}H-caffeine	$C_8H_{10}N_4O_2$	194.19	-0.55	1.0 µCi·mL^{-1}	American Radiolabeled Chemicals, St. Louis, USA
^{3}H-propranolol hydrochloride	$C_{16}H_{21}NO_2$·HCl	295.80	2.58	0.5 µCi·mL^{-1}; 10 µg·mL^{-1}	GE Healthcare, Buckinghamshire, UK
Benzalkonium chloride	$C_{25}H_{46}ClN$	396.092	1.9	1000 µg·mL^{-1}	Sigma-Aldrich, Steinheim, Germany
Methylparaben	$C_8H_8O_3$	152.15	1.67		
Thiomersal	$C_9H_{10}HgO_2S$	382.83	2.35	200 µg·mL^{-1}	
Rhodamine 123	$C_{21}H_{17}ClN_2O_3$	380.82	4.01	1.9 µg·mL^{-1}	
^{14}C-mannitol	$C_6H_{14}O_6$	182.17	-3.73	1.0 µCi·mL^{-1}	Hartman Analytic, Braunschweig, Germany
^{3}H-estriol	$C_{18}H_{24}O_3$	288.38	2.67		
^{3}H-methotrexate disodium	$C_{20}H_{22}N_8Na_2O_5$	498.40	-0.24		
^{3}H-metoprolol	$C_{15}H_{25}NO_3$	270.00	1.76		
^{14}C-nicotine	$C_{10}H_{14}N_2$	162.24	1.16		
^{14}C-urea	CH_4N_2O	60.06	-1.36		

*Obtained from http://www.chemicalize.org (data accessed on 12.07.2016), except for benzalkonium chloride (Shokri *et al.,* 2001)

3.1.3. Cell culture medium and equipment

The cell culture medium used for cultivating the HCK and HCE-T cells and the HC construct was keratinocyte growth medium (KGM): the keratinocyte basal medium (KBM™) (Lonza, St.Louis, USA) supplemented with SingleQuots™ (Lonza, St.Louis, USA) and 1 mL of 250 mM $CaCl_2$ 96% extra pure powder anhydrous (Fisher Scientific, Geel, Belgium). The prepared KGM was stored at +4°C, wrapped in aluminum foil to protect it from light. The materials, further substances and pieces of equipment, which were used are mentioned in the following sections.

3.1.4. Preparation of solutions for cell cultures

Soybean trypsin inhibitor solution: About 100 mg of soybean trypsin inhibitor (Invitrogen Corporation, Scotland, UK) was dissolved in 100 mL PBS. The solution was sterile filtered and stored at -20°C.

KGM-freezing solution: 5 mL of KGM was placed into 15 mL centrifuge tube and mixed with 2 mL of sterilized dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany) and 3 mL of fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany). This mixture was stored until use at -18°C until used for the experiments.

Collagen solution from Sigma-Aldrich (2.40 mg·mL^{-1}): Type I rat tail collagen (Sigma-Aldrich, Steinheim, Germany) was weighed and placed into a 50 mL centrifuge tube. The collagen was mixed with the appropriate amount of sterile-filtered 0.05% acetic acid (Sigma-Aldrich, Steinheim) until reaching a concentration of 2.40 mg·mL^{-1}. It was dissolved through slight rotational movement overnight at room temperature and, subsequently, it was stored at 4°C.

Solution of 10x MEM, L-glutamine (12.9 mM) and $NaHCO_3$ (16.1 mg·mL^{-1}): 22 mL of 10x MEM with Earle's salts (Biochrom AG, Berlin, Germany), 2 mL L-glutamine solution 200 mM (Sigma-Aldrich, Steinheim, Germany) and 7 mL of 71.2 mg·mL^{-1} $NaHCO_3$ (Merck, Darmstadt, Germany) were transferred into a 50 mL centrifuge tube. The solution was carefully mixed, sterile filtered and aliquoted into 2 mL Eppendorf tubes. The solution was stored at -18°C.

3.1.5. Preparation of buffer solutions

Krebs-Ringer bicarbonate-buffer (KRB) was prepared with 1.10 mM $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 1.25 mM $CaCl_2 \cdot 6H_2O$ (Sigma-Aldrich, Taufkirchen, Germany), 114.00 mM NaCl (Merck, Darmstadt, Germany), 5.00 mM KCl (Merck, Darmstadt, Germany), 1.65 mM $Na_2HPO_4 \cdot 2H_2O$, 0.30 mM $NaH_2PO_4 \cdot 2H_2O$ (Merck, Darmstadt, Germany), 20.00 mM $NaHCO_3$, 25.00 mM (D)-glucose anhydrous (Merck, Darmstadt, Germany) and 10.00 mM HEPES (Biochrom AG, Berlin, Germany). The solution was adjusted to a final pH of 7.4

(unless otherwise mentioned in the text) with H_3PO_4 (Merck, Darmstadt, Germany) or NaOH (Merck, Darmstadt, Germany).

The citrate buffer at pH 4.5, the acetate buffer at pH 5.5 and the borate buffer at pH 8.0 were prepared at 10 mM following the specification according to USP 29. The pH of the buffer solutions was adjusted with NaOH. Citric acid monohydrate ($C_6H_8O_7$) and boric acid (H_3BO_3) were obtained from Sigma-Aldrich (Steinheim, Germany). Sodium acetate ($C_2H_3NaO_2$) was purchased from Merck (Darmstadt, Germany).

Hanks balance salt solution (HBSS) at pH 6.5 contained 0.812 mM $MgSO_4$ (Sigma-Aldrich, Steinheim, Germany), 0.952 mM $CaCl_2 \cdot 6H_2O$, 126.62 mM NaCl, 5.36 mM KCl, 0.385 mM $Na_2HPO_4 \cdot 2H_2O$, 0.441 mM $K_2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany), 25 mM (D)-glucose anhydrous and 10 mM MES (Carl Roth, Karlsruhe, Germany).

HBSS at pH 7.4 was prepared with 0.812 mM $MgSO_4$, 0.952 mM $CaCl_2 \cdot 6H_2O$, 126.62 mM NaCl, 5.36 mM KCl, 0.385 mM $Na_2HPO_4 \cdot 2H_2O$, 0.441 mM $K_2HPO_4 \cdot 3H_2O$, 25 mM (D)-glucose anhydrous and 10 mM HEPES.

sofZia® at pH 7.9 contained 113.21 mM H_3BO_3, 18.82 mM NaCl, 4.02 mM KCl, 0.06 mM $ZnCl_2$, 49.53 mM 2-Amino-1-methyl-1-propanol, which were acquired from Sigma-Aldrich (Steinheim, Germany) and 1.13 mM $MgCl_2 \cdot 6H_2O$, 1.28 mM $CaCl_2 \cdot 6H_2O$, 10 mM PEG-400, 76.85 mM sorbitol, which were purchased from Merck (Darmstadt, Germany). The original composition from Chowhan *et al.,* 2010 was adapted for the current investigation.

3.2. Methods

3.2.1. Cultivation of corneal cells: HCE-T and HCK cell lines

3.2.1.1 Cell culture method provided by TUBS for the HCE-T cell line

All reagents and cell culture media were prewarmed in a water-bath at 37 °C. The HCE-T cells were initially cultivated with KGM at 37°C, 5% CO_2 and at a relative humidity of 100%. They were transferred in 25 cm² cell culture flasks (TPP, Trasadingen, Switzerland) and cultivated until reaching confluence. The cell culture medium was changed three times a week. Before starting the subcultivation of cells, an inverted phase contrast microscope was used to monitor the cell confluence and to confirm the absence of microbial contamination. Afterwards, the cell culture medium was removed from the cell culture flask and the cell monolayers were immediately rinsed carefully twice with 7 mL calcium and magnesium free PBS (Biochrom AG, Berlin, Germany). After rinsing, 1.5 mL of EDTA 2.0% in calcium and magnesium free PBS (MP Biomedicals, Illkirch, France) was added to the cells and equally distributed. The cell culture flask was incubated at 37 °C for 2 min. The EDTA solution was removed and the cells were immediately incubated with 2.0 mL of UV-inactivated trypsin-EDTA (0.05% - 0.022%) (PAA Laboratories, Cölbe, Germany) for 8 min at 37°C. The

enzymatic reaction was stopped by adding 2.0 mL of soybean trypsin inhibitor. The cell suspension was pipetted up and down, quickly transferred into a 15 mL centrifuge tube and centrifuged for 2 min at 3,694 rpm. Thereafter, the supernatant was removed and cells were resuspended in 3.0 mL of KGM by pipetting up and down. 100 µL of the cell suspension was mixed with 900 µL of 0.5% trypan blue in physiological saline (Biochrom AG, Berlin, Germany) and was counted as well as assessed with regard to cell vitality using a Fuchs-Rosenthal cell chamber (Brand, Wertheim, Germany). 150,000 HCE-T cells were seeded in a 25 cm² cell culture flask containing 7.0 mL KGM.
According to the methods provided by TUBS, a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany) should be used within the scope of the cell harvesting. As this device was not available at ACB, a Fuchs-Rosenthal cell chamber was used instead.

3.2.1.2 Cell culture method provided by TUBS for the HCK cell line

All reagents and cell culture media were prewarmed in a water-bath at 37 °C. The HCK cells were initially cultivated with KGM at 37 °C, 5% CO_2 and at a relative humidity of 100%. The cells were placed in 25 cm² cell culture flasks and cultivated until reaching confluence. The cell culture medium was changed three times a week. Before starting the trypsinization of cells, an inverted phase contrast microscope was used to monitor the cell confluence and to confirm the absence of microbial contamination. Afterwards, the cell culture medium was removed from the cell culture flask and the cell monolayers were immediately carefully rinsed twice with 7 mL PBS solution. After rinsing, 2.0 mL of UV-inactivated trypsin-EDTA was added to the cells and equally distributed. The cell culture flask was placed into the incubator for 4 min. The enzymatic reaction was stopped by adding 2.0 mL of soybean trypsin inhibitor. The cell suspension was pipetted up and down, quickly transferred in 15 mL centrifuge tube and centrifuged for 2 min at 3,694 rpm. Thereafter, the supernatant was removed and cells were resuspended in 3.0 mL KGM by pipetting up and down. 100 µL of the cell suspension was mixed with 900 µL of 0.5% trypan blue solution and the cells were counted using a Fuchs-Rosenthal cell chamber. 150,000 HCK cells were seeded in a 25 cm² cell culture flask containing 7.0 mL KGM.

3.2.1.3 Cell culture method for the HCE-T cell line after the transfer phase

The following amounts and parameters were adapted to cultivate the cells at ACB (see section 3.2.1.1). The cell monolayers were carefully rinsed twice with 15 mL PBS. UV-inactivated trypsin-EDTA (0.05% - 0.022%) was replaced by trypsin-EDTA (0.05% - 0.022%) (Biochrom AG, Berlin, Germany). The amount of 3.5 mL of EDTA 2.0% in PBS, 3.5 mL trypsin-EDTA and 3.5 mL soybean trypsin inhibitor were used. The speed of centrifugation was reduced from 3,694 rpm to 2,900 rpm. The HCE-T cells were seeded at a density of

between 200,000 and 250,000 cells in 75 cm² cell culture flasks (TPP, Trasadingen, Switzerland) and cultivated with 15 mL KGM.

3.2.1.4 Cell culture method for the HCK cell line after the transfer phase

The following amounts and parameters were adapted to cultivate the cells at ACB (see section 3.2.1.2). The cell monolayers were carefully rinsed twice with 15 mL PBS solution. UV-inactivated trypsin-EDTA was replaced by trypsin-EDTA. 3.5 mL of trypsin-EDTA and 3.5 mL soybean trypsin inhibitor were used. The centrifugation speed was reduced from 3,694 rpm to 2,900 rpm. Enzymatic digestion was reduced from 4 min to 2 min. The HCK cells were seeded at a density of between 60,000 and 100,000 cells in 75 cm².

3.2.2. Cryopreservation

One day before freezing the cells the freezing container was filled up to the mark with isopropanol (Merck, Darmstadt, Germany) and stored at -20°C. The freezing solution was diluted in cold KGM (50:50) and cooled at 4 °C for 10 min. The cold freezing solution was kept in the refrigerator all the time (at approximately 4 °C). Following subcultivation, cells were centrifuged (2 min at 2,900 rpm) and resuspended in cold freezing medium-KGM (50:50) to receive approximately 700,000 to 1,000,000 cells·mL^{-1}. 1 mL of the cell suspension was added to a labeled cryovial. The cryovials were transferred into the freezing container (a plastic box containing isopropanol to achieve a cooling rate of -1 °C min^{-1}), and were then stored at -80 °C for 24 hours. Finally, the cryovials were transferred into a tank containing liquid nitrogen at -196°C.

3.2.3. Thawing of cell lines

A cryovial was taken out of the liquid nitrogen tank and thawed by holding it in the hand until a small crystal was left under laminar air flow and sterile conditions. The cell suspension was transferred into a centrifuge tube prefilled with 15 mL cold culture medium. The cell suspension was centrifuged for 2 min at 2,900 rpm. The supernatant was discharged and the pellet resuspended with 5 mL prewarmed KGM. The suspension was transferred into 25 cm² cell culture flask containing 7 mL KGM. The cells were incubated at 37 °C and 5% CO_2. After two days, the cell culture media was replaced.

3.2.4. Cultivation of the HC construct

In vitro stromal equivalent (day 1): First, 2,782 µL of a solution of type I rat tail collagen (approximately 2.4 mg·mL^{-1} in 0.05% acetic acid) was diluted with 1,185 µL of 0.05% acetic acid (final concentration of collagen 1.7 mg·mL^{-1}). Second, the HCK cells were subcultivated; about 1,120,000 cells were resuspended in 835 µl fresh KGM. Then, 799 µL of a solution of

10x MEM, L-glutamine (12.9 mM) and $NaHCO_3$ (16.1 $mg \cdot mL^{-1}$) was added and mixed several times. This cell suspension was then mixed with the collagen solution. The final cell suspension was mixed by gently pipetting up and down until reaching homogenization. 400 µL of this cell containing collagen solution was scattered on polycarbonate Transwell® inserts with a pore diameter of 0.4 µm or 3.0 µm and a filter surface of 1.12 cm^2 (Corning Inc., New York, USA) resulting in approximately 80,000 $cells \cdot well^{-1}$. After ca.10 min (time of gel solidification), 1.5 mL of KGM was added to the basolateral side of the Transwell® inserts. Then, they were incubated at 37 °C and 5% CO_2 for 1 hour in order to complete the solidification of the gel.

Human in vitro corneal epithelium (day 1): The HCE-T cells were subcultivated and about 1,400,000 cells were resuspended in 5,600 µL of new KGM. 400 µL of the cell suspension (about 100,000 cells) was carefully poured onto the stromal equivalent. The HC construct was incubated at 37 °C and 5% CO_2. The KGM was changed as follows: The KGM was removed and 0.5 mL of KGM was added to the apical side and 1.5 mL of KGM to the basolateral side of the Transwell® inserts under the liquid-covered conditions (LCC). The KGM was replaced on day 4, 6 and 7.

ALI (Air-liquid interface) (day 7): To promote the epithelial multilayer formation, cells were exposed to ALI conditions by reducing the KGM level until the cellular surface was no longer medium. For this purpose, a metal plate was placed under the Transwell® inserts. 2.0 mL of KGM was added to the basolateral side and the KGM was changed on a daily basis from day 7 until the day of the transport experiment. The TEER were measured during cultivation with an electrical voltohmmeter, which was equipped with a chopstick electrode (see 3.2.5). The TEER of the HC construct were calculated according to Equation 3-1. The experiments were conducted on day 9, 10 and day 11.

3.2.5. *In vitro* permeability studies and transepithelial electrical resistance (TEER) measurements

Transcorneal permeability studies and TEER measurements were performed to evaluate the barrier function of the reconstructed corneal barrier during and at the end of the cultivation phase and to investigate the effect of formulation factors (pH, EDTA, calcium and preservatives) on the barrier function of the HC construct.

The TEER values provided instant information on the differentiation state of the epithelium during the cultivation phase as well as before, during and after permeation experiments. The TEER values were determined in cell culture medium (TEER in medium) with an electrical voltohmmeter (EVOM) (World Precision Instrument, Sarasota, USA), which was equipped with a chopstick electrode (STX-3).

The permeability studies were started by measuring the initial TEER of HC construct. Afterwards, the HC construct was rinsed with KRB to remove the cell culture medium. The donor solutions (drug substances in KRB, see Tables 3-2 and 3-4) were applied to the apical compartments; fresh KRB (pH 7.4) was filled into the acceptor compartments. Subsequently, the cells were equilibrated at 37 °C and 5% CO_2 for a maximum of 60 min. Following the preincubation time, the TEER values were monitored to investigate the effect of the different substances (effect of the buffer solutions and different concentrations of preservatives). After this preincubation time, the transport experiment was started in order to calculate the P_{app} value by taking samples from the donor and acceptor compartments. For this purpose, 10 µL (fluorescence measurements) and 100 µL (scintillation and HPLC measurements) from the apical side were drawn to determine the initial concentration of the donor solution. At 60, 90, 150, 210, 270, 330 and 390 min, samples of 100 µL were taken from the acceptor compartments and were immediately replaced by fresh prewarmed KRB. The TEER values were also measured during transport studies in order to evaluate the effect of the applied solutions (unless otherwise mentioned in the text). The cell models were incubated at 37 °C and 5% CO_2. All experiments were performed in triplicate and repeated twice (unless otherwise mentioned in the text). At the end of the transport experiment, the TEER of the HC construct were again measured and calculated according to Equation 3-1.

Equation 3-1:

$$TEER = R_{c(A)} = \left(R_{c+f} - R_f\right) \cdot A \text{ [Ohm·cm²]}$$

- $R_{c(A)}$ Electrical resistance of the stromal layer and stratified epithelium covering the area A [Ohm·cm^2]
- R_{c+f} Electrical resistance of the stromal layer and stratified epithelium including the filter [Ohm]
- R_f Electrical resistance of the Transwell® inserts without the stromal layer and stratified epithelium [Ohm]
- A Area of the filter insert [cm^2]

The TEER values achieved with the different transport solutions during permeation studies or incubation were normalized to the initial mean value measured in cell culture medium. These values are expressed as TEER [%] as described in Equation 3-2.

Equation 3-2:

$$TEER\ [\%] = \frac{TEER_{xtime}}{\underset{Average}{TEER_{Medium}}} \cdot 1000$$

The $TEER_{Medium}$ is the initial value of the cell culture medium, the $TEER_{xtime}$ describes the values measured at specific points in time (e.g. 10, 20, 30, 60, etc.). The influence or modification of the TEER values (IF [%]) was further calculated as described in Equation 3-3. These values were statistically analyzed and compared with the control values obtained with KRB after the preincubation time of 60 min and at the end of the permeability assays after 390 min.

Equation 3-3:

$$IF\ [\%] = 100[\%] - TEER\ [\%]$$

At the end of the drug absorption studies, the P_{app} values achieved with the different substances were calculated according to Equation 3-4,

Equation 3-4:

$$P_{app} = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{m_0} \cdot \frac{1}{A} \cdot V_D\ [cm \cdot s^{-1}]$$

where Q/t is the permeation rate (steady state transport rate) obtained from the profile of the transported amount of substrate versus time, calculated by the linear regression of time and concentration. A represents the exposed area of the stratified epithelium (1.12 cm^2 on Transwell® inserts). m_0 is the initial mass of test compound in the donor compartment, and V_D is the buffer volume of the donor compartment [cm^3].

3.2.6. Determination of pH

The pH values of the buffer solutions were measured with the pH meter PerpHecT Meter Orion model 330 (Orion Research, Boston, USA). This instrument was calibrated with a two-point curve bracketing the expected pH values.

3.2.7. Determination of osmolality

The osmolality measurement of the buffer solutions was performed using an OSMOMAT® 030 (Gonotec, Berlin, Germany). The instrument was calibrated with distilled water and a calibration standard of 300 $mOsmol \cdot kg^{-1}$ was used (Gonotec, Berlin, Germany).

3.2.8. Evaluation of preservatives

First, the TEER values were measured (TEER in medium). The cell culture medium was removed and the HC construct was rinsed with KRB to remove the cell culture medium. 500 µL of the donor solution was applied to the donor compartments; fresh KRB (pH 7.4) was filled into the acceptor compartments. Subsequently, the cells were incubated at 37 °C and 5% CO_2. The TEER values were measured every 10 min for 30 min in order to investigate the effect of preservatives at different concentrations. After this time, the TEER values were continuously measured after 60, 90, 150, 210, 270, 330, and 390 min.

3.2.9. Quantification of samples

3.2.9.1 Quantification of samples using fluorescence spectroscopy

The components of the fluorescence system consisted of a Wallac Victor™ Multilabel Counter fluorescence plate reader (PerkinElmer Wallac, Freiburg, Germany) equipped with a photomultiplier detector and excitation and emission filters of 485 nm and 535 nm for sodium fluorescein, rhodamine 123 and fluorescein isothiocyanate dextran. The output signal was monitored and processed using the Wallac 1420 Multilabel Counter software. A fluorescence plate reader (Tecan, Männerdorf, Switzerland) was also used for rhodamine B. These samples were measured by the Department of Dermatology and Venereology of the University Hospital Hamburg-Eppendorf (UKE) using excitation and emission filters of 535 nm and 590. In order to avoid shortcomings, the concentration of each transport solution with Na-FLU at different pH values was determined by running individual calibration curves, which resulted in a correlation coefficient of at least 0.995. The employed analytical methods correspond to the acceptance criteria recommended by the applicable guidelines (ICH, 2005; FDA Guidance For Industry, 2001).

3.2.9.2 Quantification of samples using liquid scintillation counting

The radiolabeled samples were measured with a Wallac 1450 Microbeta Trilux ™ scintillation counter (PerkinElmer Wallac, Freiburg, Germany) after the addition of the scintillation cocktail OptiPhase SuperMix (PerkinElmer, Waltham, USA). The calibration curve was in the range of the evaluated concentrations with a correlation coefficient of ≥ 0.995 and met the acceptance criteria recommended by the applicable guidelines (ICH, 2005; FDA Guidance For Industry, 2001).

3.2.9.3 Quantification of samples using HPLC

Timolol maleate: The HPLC system used was a Waters Alliance 2487 (Waters, Milford, MA, USA), which was equipped with a quaternary pump, an autosampler and a dual absorbance detector. The output signal was monitored and processed using the Empower™ 2 software. The absorption was monitored at 295 nm. The chromatographic column used was a Waters Symmetry ™ C8, 150 mm x 3.9 mm, 5.0 µm. An isocratic method was used. The mobile phase contained 12.5% (v/v) acetonitrile (Merck, Darmstadt, Germany) and 87.5% (v/v) H_2O with 0.1% triethylamine (Sigma-Aldrich, Steinheim, Germany) and was adjusted to a pH of 2.9 using anhydrous acetic acid. The flow rate was 1.2 $mL \cdot min^{-1}$. The column temperature was set at 40 °C. The autosampler was maintained at 20 °C and the injection volume was 20 µL. The calibration curve was in the range of 0.515 $\mu g \cdot mL^{-1}$ – 98.689 $\mu g \cdot mL^{-1}$ with a correlation coefficient ≥ 0.995.

Benzalkonium chloride: The HPLC system used was an Agilent 1100 series (Agilent Technology, Hewlett Packard, Waldbronn, Germany), which was equipped with a binary pump, an autosampler and an absorbance detector. The output signal was monitored and processed using the ChemeStation software. The absorption was monitored at 210 nm. The chromatographic column used was a Waters Spherisorb CN, 150 mm x 4.6 mm, 5.0 μm or Phenomenex 150 mm x 4.6 mm, 3.0 μm. The mobile phase contained a gradient mixture of 0.02 M potassium dihydrogen phosphate buffer (Merck, Darmstadt, Germany) pH 5.5 (eluent A) and acetonitrile (eluent B). The gradient method used consisted of decreasing the concentration of eluent A from 6:4 (A - B) to 3:7 until 7 min. This level was maintained for 2 min and then again increased to 6:4 (A - B) until 11 min. The flow rate was 1.2 $mL \cdot min^{-1}$ with a retention time of 5 min. The column temperature was set at 20 °C. The autosampler was maintained at 20 °C and the injection volume was 20 μL. The calibration curve was in the range of 10.21 $\mu g \cdot mL^{-1}$ – 198.6 $\mu g \cdot mL^{-1}$ with $R^2 \geq 0.995$.

Thiomersal: The HPLC system used was an Agilent 1100 series (Agilent Technology, Hewlett Packard, Waldbronn, Germany), which was equipped with a binary pump, an autosampler and an absorbance detector. The output signal was monitored and processed using the ChemeStation software. The absorption was monitored at 222 nm. The chromatographic column used was an X Terra, 150 mm x 4.6 mm, 5.0 μm. An isocratic method was used. The mobile phase contained 78.0% (v/v) of 0.02 M potassium dihydrogen phosphate buffer pH 5.5 and 22.0% (v/v) acetonitrile. The flow rate was 1.0 $mL \cdot min^{-1}$ with a retention time of 4 min. The column temperature was set at 20 °C. The autosampler was maintained at 20 °C and the injection volume was 20 μL. The calibration curve was in the range of 1.00 $\mu g \cdot mL^{-1}$ – 200.96 $\mu g \cdot mL^{-1}$ with $R^2 \geq 0.995$.

Methylparaben: The HPLC system used was an Agilent 1100 series (Agilent Technology, Hewlett Packard, Waldbronn, Germany), which was equipped with a binary pump, an autosampler and an absorbance detector. The output signal was monitored and processed using the ChemeStation software. The absorption was monitored at 215 nm. The chromatographic column used was a Luna, 150 mm x 4.6 mm, 3.0 μm. The mobile phase was 65.0% (v/v) of 0.02 M potassium dihydrogen phosphate buffer (pH 5.5) and 35.0% (v/v) acetonitrile. The flow rate was 1.0 $mL \cdot min^{-1}$ with a retention time of 5 min. The column temperature was set at 20 °C. The autosampler was maintained at 20 °C and the injection volume was 20 μL. The calibration curve was in the range of 0.195 $\mu g \cdot mL^{-1}$ – 305.3 $\mu g \cdot mL^{-1}$ with $R^2 \geq 0.995$.

Propranolol: The HPLC system used was an Agilent 1200 series (Agilent Technology, Hewlett Packard, Waldbronn, Germany), which was equipped with a binary pump, an

autosampler and an absorbance detector (Waters, Milford, MA, USA). The output signal was monitored and processed using the Empower 2 software. The absorption was monitored at 215 nm. The chromatographic column used was a Bischoff prontosil RP8, 53 mm x 4.0 mm, 5.0 μm. The mobile phase consisted of 75.0% (v/v) of 10 mM sodium dihydrogen phosphate (Merck, Darmstadt, Germany) adjusted to a pH of 3.5 using H_3PO_4 and 25.0% (v/v) acetonitrile. The flow rate was 1.0 $mL \cdot min^{-1}$ with a retention time of 3 min. The column temperature was set at 40 °C. The autosampler was maintained at 20 °C and the injection volume was 20 μL. The calibration curve was in the range of 0.019 $\mu g \cdot mL^{-1}$ – 9.89 $\mu g \cdot mL^{-1}$ with $R^2 \geq 0.995$.

3.2.1. Data evaluation and statistics

Statistical calculations of TEER and P_{app} values were performed using the MS Excel 2010 spreadsheet package and Data Analysis, Technical Graphics Origin version 8.6 (Microcal (TM) Software, Inc, Northampton, USA). One-Way ANOVA test was performed followed by Mann Whitney test. The data were considered as significantly different at p-values *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4. Results and discussion

4.1. Transferability of methods, quality and prevalidation of the Hemicornea model

4.1.1. Transfer of cell culture conditions and quality of the Hemicornea model

The use of a corneal cell culture model for *in vitro* drug absorption studies must meet several requirements in order to be validated as an alternative system to animal cornea. This so-called prevalidation protocol consists of three main phases; i) protocol refinement, ii) protocol transfer and, iii) protocol performance (Curren *et al.,* 1995). The first phase, including development of the methods for constructing (3D) human Hemicornea model (Hemicornea, HC) or Hemicornea construct used and protocols for conducting drug absorption studies, was performed by Matthias Hahne at TUBS (Hahne and Reichl*,* 2011). The present chapter describes the protocol transfer and performance for the prevalidation of the HC construct done at ACB. Subsequently, the data was evaluated by Matthias Hahne at TUBS and the results were summarized in Hahne *et al.,* 2012. This work was funded by the German Federal Ministry for Education and Research (grant no. 0315504C).

The HC construct employed in this investigation consists of immortalized HCK cells embedded into a collagen stromal matrix (Zorn-Kruppa *et al.,* 2005) cast into Transwell® inserts and immortalized HCE-T cells (Araki-Sasaki *et al.,* 1995) seeded onto the collagen matrix (see 3.2.4). The HCE-T cell line is until today most commonly used to build corneal models due to its capacity to display morphological characteristic similar to those of the human cornea (Toropainen *et al.,* 2001; Becker *et al.,* 2007; Seeber *et al.,* 2008; Reichl *et al.,* 2011), whereas co-cultivation with HCK cells provides the HC construct with more *in vivo* properties since they influence the morphology of the corneal epithelium (Orwin and Hubel*,* 2000). The first step to develop an *in vitro* corneal model is the cultivation of different cell types. For the current investigation, HCK and HCE-T cells were first cultivated in 25 cm² and then in 75 cm² culture flasks in a 5% CO_2 atmosphere at 37 °C until the confluence of cell monolayers. This required minor modifications in the seeding density of cells in order to reach confluence once a week. The light microscopic examination of HCK cells revealed that this cell type exhibited a spindle-like and flattened morphological shape as reported by Zorn-Kruppa *et al.,* 2005, whereas HCE-T cells showed a cobblestone-like appearance. This morphological shape has previously been described by Araki-Sasaki *et al.,* 1995.

The HC construct was cultivated under LCC until day 7 (see Figure 4-1), which was found to be an optimal day to expose the epithelial confluent monolayer to the ALI conditions (Hahne and Reichl*,* 2011) and to simulate *in vivo* conditions (Chang *et al.,* 2000). The TEER values

declined sharply between day 4 and 7 probably due to nutrient depletion in the growth medium. After the culture medium was renewed and the HC construct was exposed to ALI conditions, the transepithelial electrical resistances (TEER) increased abruptly within 24 hours and only modestly between day 9 and 11. Thereafter, a gradual decrease in the TEER values was observed (data not included). TEER values between 300 - 600 Ohm·cm^2 have been suggested to reflect sufficient tightness of the epithelial cell layers for drug absorption studies (Steimer *et al.,* 2005; Becker*,* 2006; Hahne and Reichl*,* 2011). The TEER values obtained in the present study achieved compliance with the acceptance limits and thus, it can be concluded that the transfer of methods for constructing the HC construct was successfully performed at ACB.

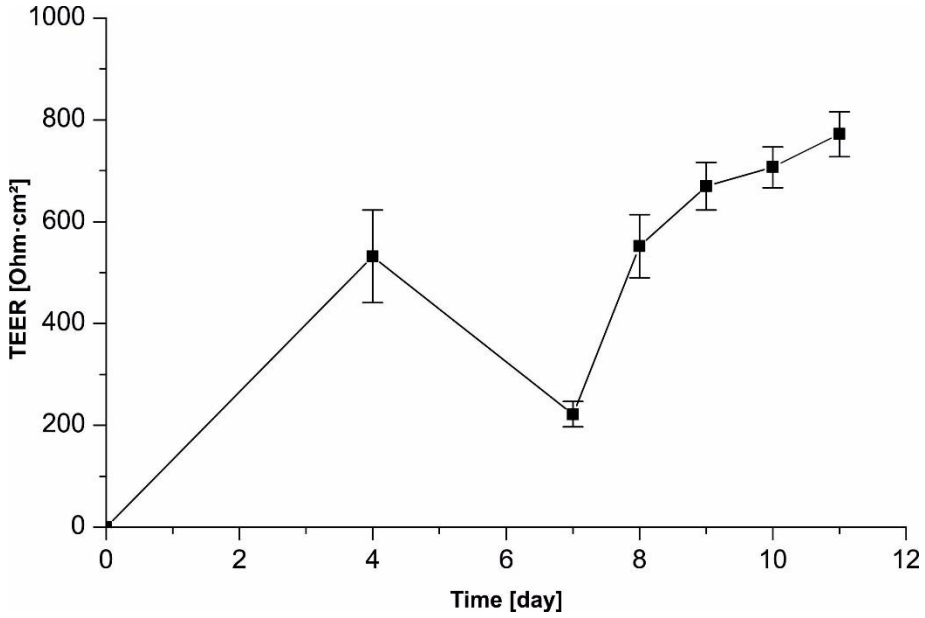

Figure 4-1: TEER (mean ± SD) reached under LCC until day 7 and after the HC construct was exposed to *ALI* conditions in a serum-free KGM (n = 6)

In accordance with the description provided in section 3.2.5, the transport studies were carried out with sodium fluorescein (Na-FLU), which is a hydrophilic permeation marker. The estimation of the TEER and permeation coefficients (P_{app}) values for the HC construct was performed as described in section 3.2.5. For the purpose of evaluating the acceptance criteria for the transfer phase previously defined P_{app} of Na-FLU < $1 \cdot 10^{-6}$ cm·s^{-1} and TEER values ≥ 400 Ohm·cm² (Hahne and Reichl*,* 2011), the P_{app} values of Na-FLU were plotted against the TEER values obtained after the preincubation time (see Figure 4-2). The P_{app} data obtained with Na-FLU for the HC construct shows that at a minimum TEER value of 400 Ohm·cm² P_{app} values below the defined acceptance criteria (quality criterion) are achieved (vertical dotted line, Figure 4-2). In summary, the values obtained with Na-FLU are an indicator for a successful transferability of the protocols for drug absorption studies and fulfill the acceptance criteria established already in TUBS.

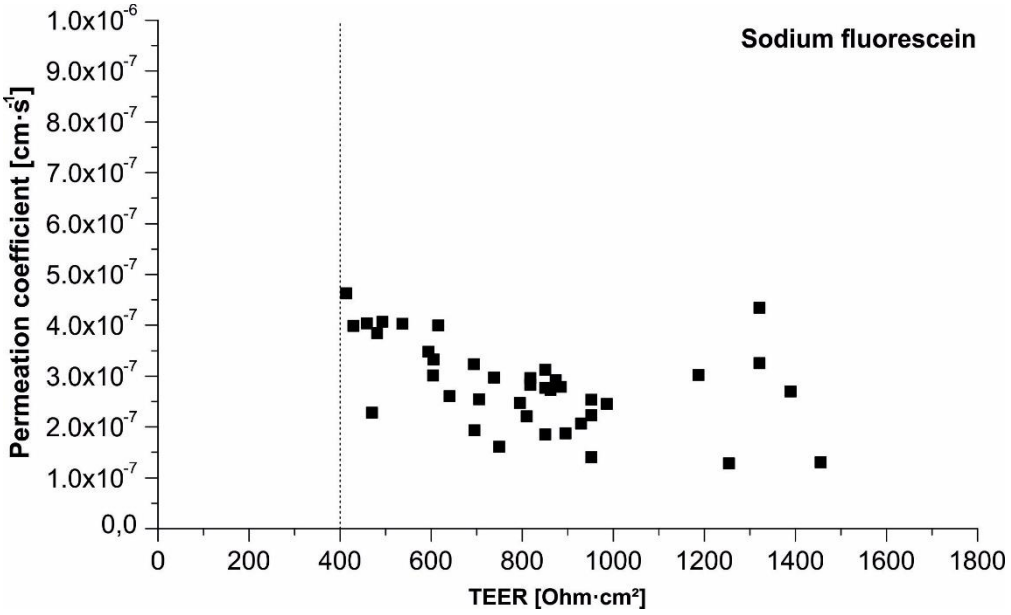

Figure 4-2: TEER and P_{app} values obtained with Na-FLU for the HC construct resulting in n=39 (6 independent experiments) during the transfer phase. The vertical dotted line represents the minimum TEER value obtained for the HC construct.

4.1.2. Prevalidation of the HC construct

After the successful transfer of the cultivation and permeation protocols and the compliance with acceptance criteria was achieved, the intralaboratory and interlaboratory reproducibility of the HC construct was evaluated. For this purpose, the permeation of some molecules with different molecular properties was investigated (e.g. fluorescein isothiocyanate dextran (FD-4), a hydrophilic macromolecule and rhodamine B, a lipophilic substance) (Figure 4-3). The same permeation markers together with Na-FLU were used by all involved laboratories. The P_{app} values for the different permeation markers versus the TEER values obtained were then compared to those measured in the other laboratories (Figure 4-3). The TEER values obtained at TUBS were between 380 and 780 Ohm·cm², the HC constructs showed TEER values between 200 and 1500 Ohm·cm² at the Department of Dermatology and Venereology of the University Hospital Hamburg-Eppendorf (UKE) and the HC constructs cultivated at ACB had TEER values between 580 and 1600 Ohm·cm². The source of variability observed for the reported TEER values may be due to differences in the methodology employed to determine TEER values and/or the levels of expression of zona occludens in the HCE-T cells grown in each laboratory.

To evaluate the transferability and to define a common acceptance criterion for the HC construct, the TEER - P_{app} correlation was evaluated based on the best-fit line. In order to determine the minimum TEER value to be used as a quality criterion of the HC construct a vertical line was drawn in the intersection point of the regression with the horizontal line corresponding to the suggested P_{app} value for the human cornea. This value corresponds to the arithmetic mean of the P_{app} measured for the rabbit and porcine cornea (Hahne and

Reichl, 2011). The intersection point for Na-FLU lies at 440 Ohm·cm² and for FD-4 at 500 Ohm·cm².

No correlation between TEER and P_{app} value was found for the lipophilic permeation marker rhodamine B (Figure 4-3). This is due to the fact that the transcorneal diffusion of the lipophilic substance depends to a considerably smaller extent on the paracellular pathway, which is reflected the TEER value (Prausnitz and Noonan, 1998). All together, these results confirmed the quality criterion (acceptance limits) defined during the transfer phase at ACB (a minimum of 400 Ohm·cm² is required).

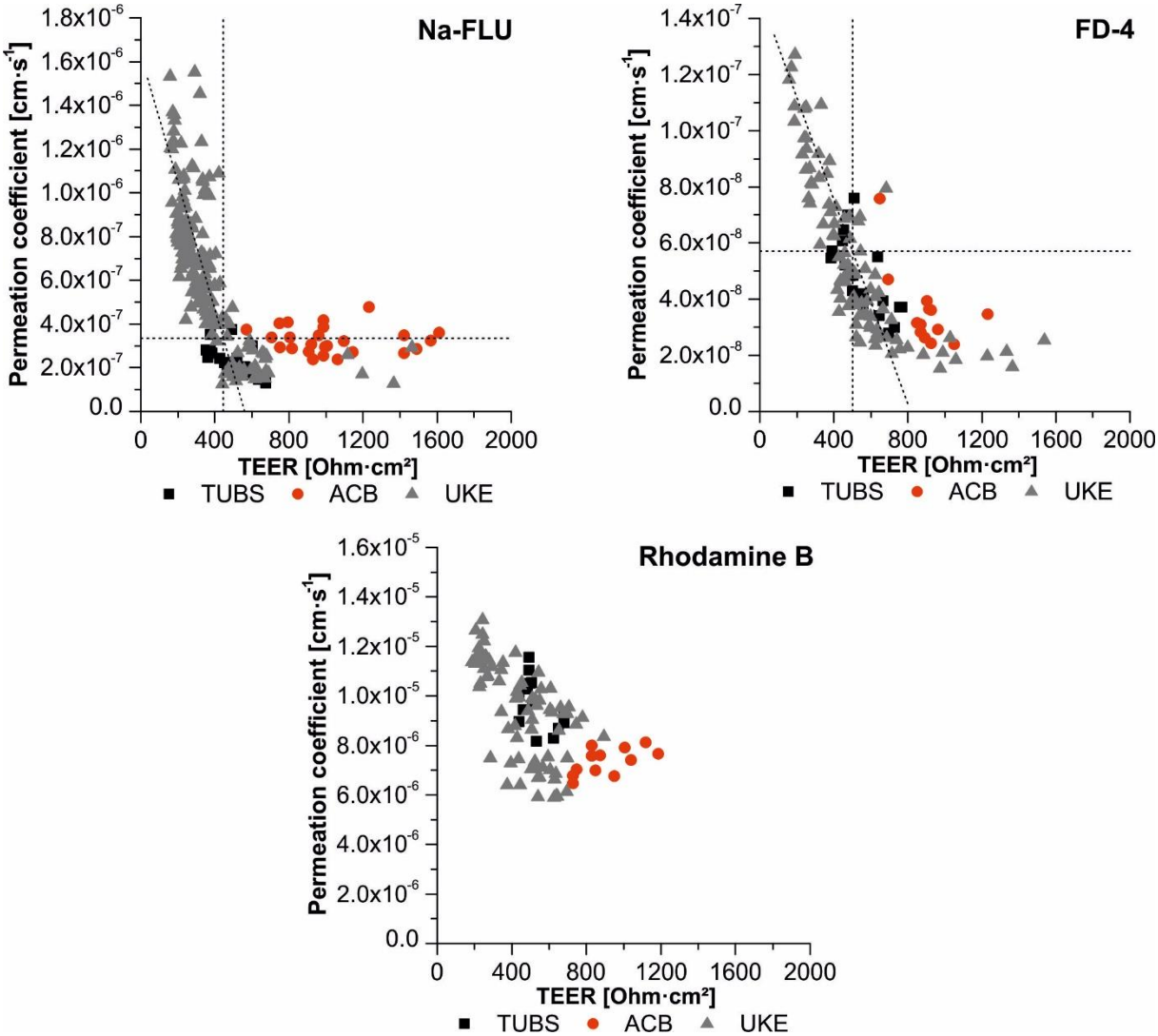

Figure 4-3: Representation of the correlation between TEER and P_{app} values for Na-FLU, FD-4 and rhodamine B during the prevalidation of the HC construct, resulting in n=85 (14 independent experiments) for Na-FLU; n=92 (16 independent experiments) for FD-4, n=66 (8 independent experiments) for rhodamine B. The minimum TEER value defined as a quality criterion for the HC construct is depicted in the graphics Na-FLU and FD-4. This value is represented by the vertical line in both graphics and was obtained from the intersection of the best-fit line (decreasing line) and the horizontal line, which is the average P_{app} value for isolated rabbit and porcine cornea (adapted from Hahne *et al.,* 2012).

The broad range of different P_{app} values of selected markers and the TEER values generated during cultivation and diffusion transport provide a great opportunity to correlate the P_{app} values of Na-FLU, FD-4 and rhodamine B with the respective TEER values in order to establish only one acceptance criterion for the evaluation of results as is represented in Figure 4-3.

In order to evaluate the reproducibility of the HC construct in each laboratory involved and its applicability for drug absorption, four relevant ophthalmic active compounds (aciclovir, bimatoprost, dexamethasone and timolol maleate) were selected and further tested in permeation studies. HC batches displaying TEER values ≥ 440 Ohm·cm² were taken for the evaluation of the intralaboratory and interlaboratory reproducibility. A summary of the mean P_{app} calculated for the permeation markers and these substances in all the laboratories is presented in Table 4-1. For comparison, the P_{app} values obtained for excised rabbit and porcine cornea by Matthias Hahne at TUBS are also shown.

Table 4-1: Summary (mean ± SD) of the P_{app} of all substances for the HC construct observed in the participating laboratories and comparison of these values with the mean P_{app} of all HC as well as with those of rabbit and porcine cornea. *Experiments were only conducted at TUBS (adapted from Hahne *et al.,* 2012).

Permeation marker/ Active drug	**P_{app} 10^{-6} ± SD [cm·s^{-1}]**					
	Mean HC TUBS	**Mean HC ACB**	**Mean HC UKE**	**Mean of all HC**	***Rabbit cornea**	***Porcine cornea**
Na-FLU	0.19 ± 0.05	0.33 ± 0.06	0.27 ± 0.08	0.24 ± 0.08	0.40 ± 0.11	0.18 ± 0.15
FD-4	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.09 ± 0.05	0.017± 0.04
Rhodamine-B	9.65 ± 1.02	7.35 ± 0.54	6.89 ± 0.76	8.40 ± 1.50	13.5 ± 2.11	5.43 ± 1.26
Aciclovir	2.87 ± 0.51	1.91 ± 0.28	1.69 ± 0.41	2.20 ± 0.68	4.04 ± 0.98	2.03 ± 0.58
Bimato-prost	2.20 ± 0.44	2.90 ± 0.98	5.12 ± 1.02	3.44 ± 1.51	3.63 ± 1.01	1.47 ± 0.45
Dexametha-sone	5.40 ± 0.46	5.91 ± 1.27	6.26 ± 0.39	5.83 ± 0.87	7.68 ± 0.87	1.51 ± 0.12
Timolol maleate	7.78 ± 0.74	7.99 ± 0.30	7.18 ± 0.60	7.63 ± 0.67	22.5 ± 3.95	7.13 ± 1.79

The analysis of the intralaboratory and interlaboratory reproducibility was performed by Hahne *et al.* using an equivalence test in which 95% confidence levels were calculated for the P_{app} value of each individual HC batch (Hahne *et al.,* 2012). The equivalence was confirmed by comparing these confidence levels with the predefined acceptance criteria for excised rabbit cornea (Figure 4-4). The acceptance limits were determined using the P_{app}

values obtained with excised rabbit cornea, which were employed as reference values since they are commonly used in similar investigations. These experiments were performed at the TUBS laboratory. The permeation marker FD-4 was employed in order to ensure that future batches meet the predefined acceptance limits and since it showed the greatest variability of the mean P_{app} values (see Table 4-1 and Figure 4-4). The three 95% confidence levels for FD-4 were obtained independently for every single batch of rabbit corneal tissue (Figure 4-4) (Hahne *et al.,* 2012). In addition, the largest deviation of these confidence levels (84.2%) compared to the mean value was specified as the relative variance (Hahne *et al.,* 2012).
This relative variance served to define the acceptance limits for HC batches. Within the scope of this prevalidation study, all studied 73 HC batches were in agreement with the acceptance limits defined for the intralaboratory reproducibility. However, with regard to the interlaboratory reproducibility, the HC batches varied slightly between the laboratories; but only 1 out of 73 sets of experiments did not meet the acceptance criteria.

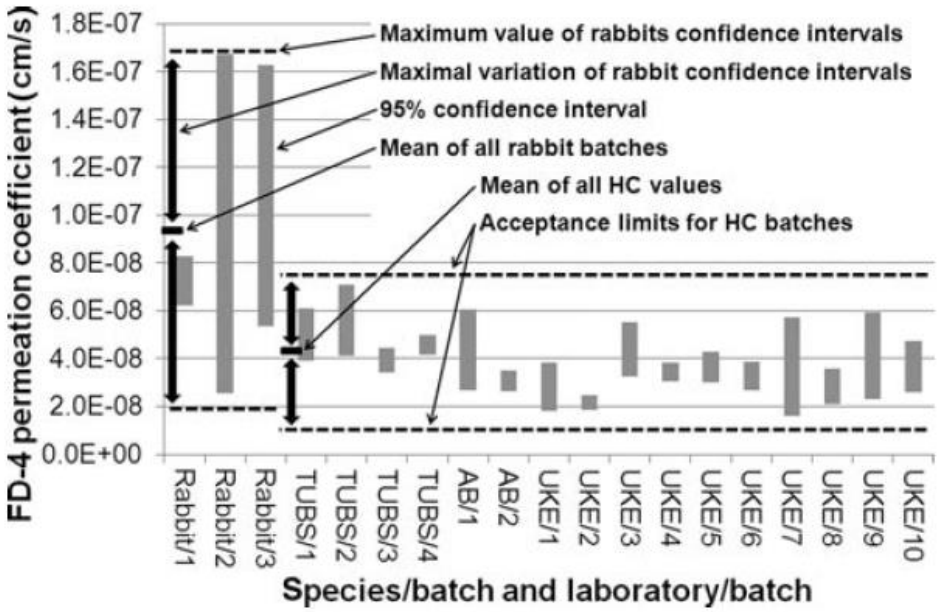

Figure 4-4: Schematic representation of the procedure for the equivalence test conducted at TUBS to evaluate the intralaboratory and interlaboratory reproducibility of the HC construct. The highest relative variability was 84.2% and was calculated based on the average values of all rabbit experiments performed at TUBS (adapted from Hahne *et al.,* 2012).

4.1.3. Summary of transferability of methods, quality and prevalidation of the HC construct

The development of cornea models for studying *in vitro* drug absorption involves several phases before they can be finally validated as an alternative to *in vitro* and *in vivo* studies with animal cornea. This chapter describes the last two phases of the prevalidation process (protocol transfer and protocol performance) for constructing the HC model and for conducting drug absorption studies, which were carried out at ACB. The protocol refinement, which is the first stage in the prevalidation of a test model was already reported and mainly

performed by Matthias Hahne at TUBS (Hahne and Reichl, 2011). While the refinement of protocols is performed in one laboratory, the transfer and performance processes must be conducted in at least two different laboratories to ensure the model's transferability and reproducibility. Within the framework of the present investigation TUBS, ACB and UKE were involved in the prevalidation process.

A prevalidation process is assessed according to predetermined acceptance criteria that are defined in relation to studies based on excised cornea either from rabbit or porcine. Following the successful prevalidation, the HC model undergoes the validation process in order to be approved by the corresponding authorities, which ensure its worldwide applicability.

The results demonstrated that the methods for developing the HC construct were successfully transferred. The TEER values of the HC construct are consistent with the formation of tight epithelial cell layers, which is a mandatory requirement that corneal models have to fulfill in order to be suitable for drug permeation studies (Reichl, 2008). The TEER values were in accordance with the acceptance TEER value defined by Matthias Hahne at TUBS.

The HC batches with acceptable TEER values were then employed in permeability studies using standard permeation markers including Na-FLU. The P_{app} values obtained for Na-FLU were within the previously defined acceptance permeation limit of the HC model (Hahne and Reichl, 2011).

In order to evaluate the intralaboratory and interlaboratory reproducibility, the P_{app} values for the different HC batches were calculated for seven substances with different physicochemical properties; the values obtained were then compared within the scope of an equivalence test as described in Hahne *et al.,* 2012. While the HC construct showed 100% intralaboratory reproducibility, the interlaboratory reproducibility decreased slightly to 98.6% (1 out of 73 HC batches did not fulfill the accepted criteria). Despite this small decrease in the reproducibility between laboratories, the HC construct still complies with the acceptance criteria defined during the prevalidation phase.

Based on this, within the scope of the present study, it has been observed that the permeability of the HC construct to substances is similar to that of excised corneal tissue, which suggests that the HC construct possesses an acceptable level of intra and interlaboratory reproducibility.

In conclusion, the present investigation resulted in the successful protocol transfer and protocol performance process for constructing a cornea model for conducting drug absorption studies.

4.2. Comparative analysis of transcorneal drug absorption

4.2.1. Differentiation capacity of the HC construct depending on the physicochemical properties of substances

The transcorneal permeation rate of an ocular drug is an important parameter, which needs to be evaluated in the development phase of ophthalmic formulations. The permeation of drugs through the corneal barrier is influenced by their physicochemical properties (Järvinen *et al.,* 1995). *In vivo* and *ex vivo* experiments with rabbit cornea are frequently conducted within the scope of such studies. However, several disadvantages have been reported with regard to these studies, such as the high variability of permeability data between laboratories, the high costs for keeping laboratory animals as well as ethical concerns (Kruszewski *et al.,* 1997; Kawazu *et al.,* 1998; Reichl *et al.,* 2004). Therefore, *in vitro* corneal models have been developed to solve these issues. In order to demonstrate that the permeation of substances through the HC construct is also influenced by their physicochemical properties, as has already been reported for native corneal tissue, the permeability of the evaluated HC construct to 18 substances was correlated with their lipophilicity (referred to as log *P*) and molecular weight (MW). Furthermore, the P_{app} values of these substances were also used to determine the degree of comparability between the permeability of the HC construct and the permeability of excised rabbit and human cornea. P_{app} values of *ex vivo* animal cornea were obtained from literature.

For a detailed evaluation of the permeability of the prevalidated HC construct, in particular, two aspects relevant for drug development (i.e. improvement of ocular bioavailability) were investigated: i) the ability of different substances to permeate the corneal barrier without excipients and ii) the permeability of the cornea model to diverse ophthalmic drugs as compared with that of excised tissue. To cover a wider range of physicochemical properties, highly hydrophilic paracellular markers (Na-FLU and mannitol) and lipophilic markers (rhodamine B) were used (Kawazu *et al.,* 1998; Hahne and Reichl*,* 2011).
The Table 4-2 provides an overview of the P_{app} of all substances obtained with the HC construct as well as their respective MW and log *P* values.

Table 4-2: Mean P_{app} values ± SD of all substances achieved with the HC construct, as well as details on their MWs and log *P*. The letter n represents the number of independently conducted experiments.

Substance	P_{app} 10^{-6} ± SD [cm·s^{-1}]	Molecular weight [g·mol^{-1}]	*Log *P*	n
FD-4	0.044 ± 0.02	4400	-1.52	12
Na-FLU	0.29 ± 0.08	376.27	-2.0	32
Mannitol	0.38 ± 0.08	182.17	-3.73	19

Substance	P_{app} 10^{-6} ± SD [cm·s^{-1}]	Molecular weight [g·mol^{-1}]	*Log *P*	n
Methotrexate disodium	1.97 ± 0.47	498.40	-0.24	6
Aciclovir	2.07 ± 0.35	225.20	-1.03	12
Bimatoprost	2.95 ± 1.04	415.57	2.63	12
Urea	2.81 ± 0.50	60.06	-1.36	6
Dexamethasone	7.38 ± 1.83	392.46	1.68	12
Timolol maleate	10.30 ± 0.42	316.42	1.34	12
Metoprolol	10.05 ± 0.57	270.00	1.76	6
Propranolol hydrochloride	12.23 ± 1.40	295.80	2.58	33
Rhodamine B	12.42 ± 2.11	479.02	1.78	12
Caffeine	7.25 ± 0.67	194.19	-0.55	6
Nicotine	10.82 ± 1.30	162.24	1.16	6
Estriol	10.38 ± 2.25	288.38	2.67	3
Benzalkonium chloride	7.33 ± 0.98	396.02	1.90	6
Methylparaben	10.76 ± 1.49	152.15	1,67	6
Thiomersal	5.68 ± 0.83	382.83	2.35	6

*Obtained from ChemSpider chemical databank (http://www.chemicalize.org, accessed at 12.07.2016), except for Na-FLU, FD-4 (Sakai *et al.,* 1997) and benzalkonium chloride (Shokri *et al.,* 2001)

The absorption rate of a drug for transcorneal transport appears to dependent on the octanol-water partition coefficient, hereinafter referred to as log *P* (Schoenwald and Huang, 1983; Wang *et al.,* 1991; Sasaki *et al.,* 1995a). The P_{app} values for the different substances were calculated as described in section 3.2.5 and plotted against the respective log *P* as listed in Table 4-2 (Figure 4-5). This graphic shows that the permeability of the HC construct sigmoidally increased for substances with increased log *P* values. The data were fitted to a sigmoidal curve with a R^2 of 0.85 for all substances. An identical relationship was also described for excised rabbit cornea with β-blockers where a R^2 of 0.84 was calculated (Wang *et al.,* 1991). According to the sigmoidal correlation, the R^2 found for the HC construct was thus in line with the value reported by Wang *et al*. This indicates that the barrier properties of the HC construct are largely equivalent to that of excised rabbit tissue. Furthermore, Figure 4-5 shows that for substances with log *P* values below -0.24, with the exception of caffeine the permeability of the HC construct is minor influenced by their hydrophilic properties (log *P* values). However, for substances with log *P* values above 0.08, a higher influence of log *P* values on the permeability of the HC construct and the variability of P_{app} values can be observed.

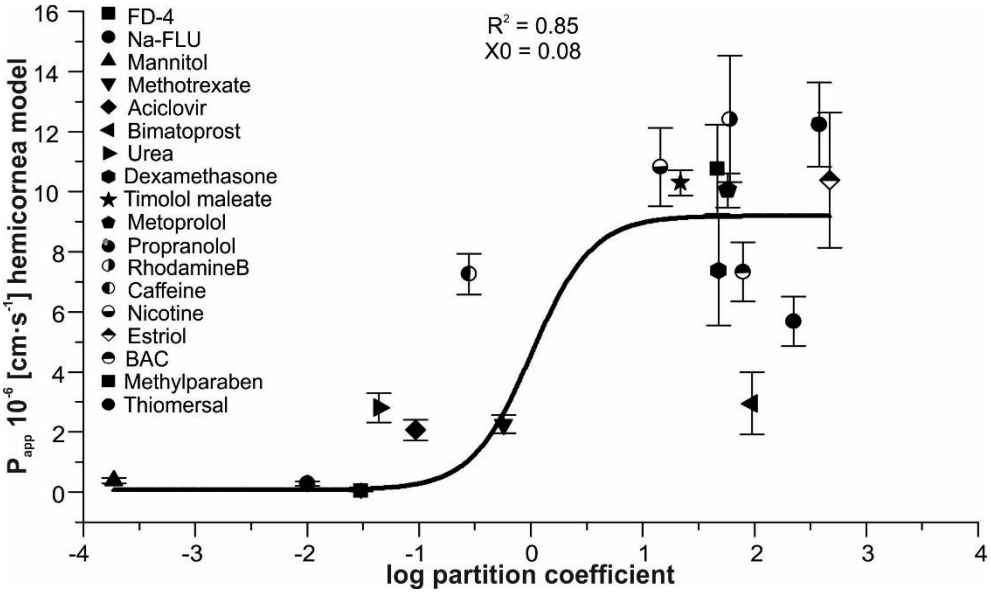

Figure 4-5: Permeability of the HC construct as a function of log *P* of the permeated substances. Each symbol represents the mean ± SD of three to six independent experiments carried out for each substance.

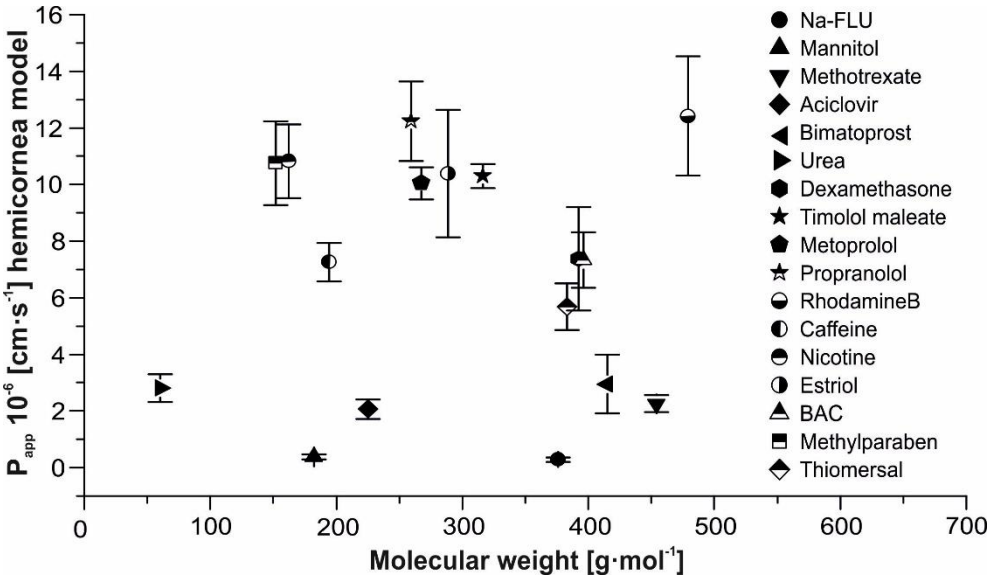

Figure 4-6: Permeability of the HC construct as a function of molecular weight of permeation markers. Each symbol represents the mean ± SD of three to six independent experiments carried out for each substance.

Another parameter is the MW as described by Prausnitz and Noonan, 1998. In Figure 4-6 the MW of 17 substances are plotted against the corresponding P_{app} values. The macromolecule FD-4 was not included in this analysis since 94% of the tested substances have a MW, which is not higher than 500 g·mol^{-1}. The graphic shows that no correlation exists between the permeability of the HC construct and the MW of permeation markers ranging from 60 to 500 g·mol^{-1}. This result is in accordance with previously reported data for isolated rabbit cornea (Prausnitz and Noonan, 1998). Prausnitz and Noonan have demonstrated in their

comprehensive study that the permeability of rabbit cornea is not influenced by the molecular size of drugs with MW mentioned above.

4.2.2. Comparison of the permeability of the HC construct with those of *ex vivo* rabbit and human cornea based on the P_{app} values

The section above describes that the HC construct was assessed with regard to its capability to discriminate between poorly or highly permeable substances. For successful use of an alternative cornea model, another important aspect, which has to be taken into account is the extent to, which its permeability is comparable to well-established excised corneal models. Therefore, in order to investigate the suitability of the HC construct for drug absorption studies, HC batches exhibiting TEER values larger than 400 Ohm (minimum TEER value accepted for permeability assay as previously defined during the transfer phase, 4.1.1) were considered. The P_{app} values for the HC construct were directly compared with the P_{app} values of excised rabbit and human cornea available from the literature (Figure 4-7 and Figure 4-8). It is worth noting that excised rabbit corneas are most commonly used for drug permeation studies (Mannermaa *et al.,* 2006; Xiang *et al.,* 2009; Reichl *et al.,* 2011).

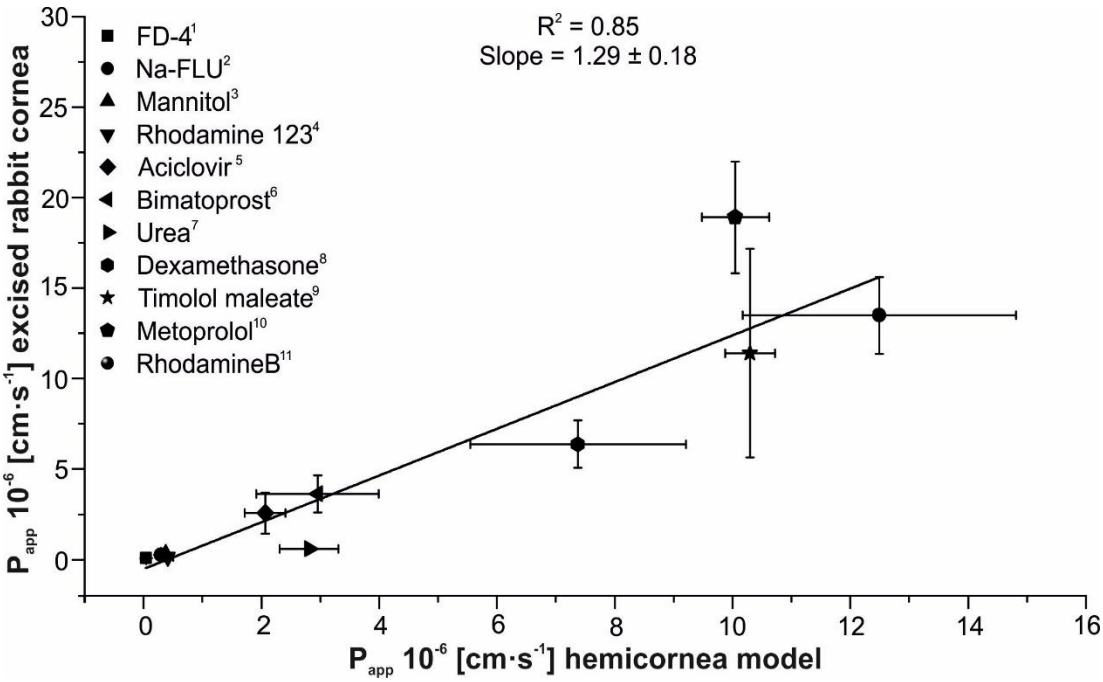

Figure 4-7: P_{app} values of substances obtained for the HC construct (n = 6-39) compared with those for the excised rabbit cornea. The numbers denote the references where the values were extracted, as follows: (1) Nakamura *et al.,* 2007; Hahne *et al.,* 2012; (2) Becker, 2006; Hahne *et al.,* 2012; (3) Reichl, 2008; Suhonen *et al.,* 1998; (4) Becker, 2006; (5) Suresh *et al.,* 2010; Hahne *et al.,* 2012; (6) Hahne *et al.,* 2012; (7) Klyce, 1975; (8) Civiale *et al.,* 2004; Hahne *et al.,* 2012; (9) Huang *et al.,* 1983; Ahmed *et al.,* 1987; Shih and Lee, 1990; Wang *et al.,* 1991; Becker, 2006; Hahne *et al.,* 2012; (10) Huang *et al.,* 1983; Xiang *et al.,* 2009); (11) Hahne *et al.,* 2012

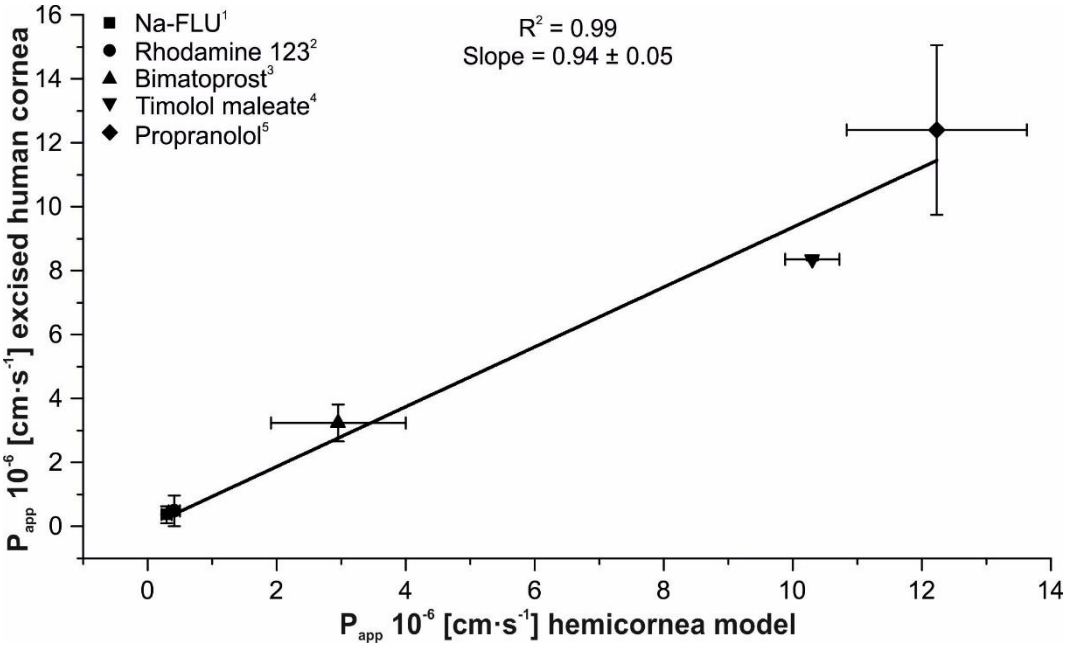

Figure 4-8: P_{app} values of substances obtained for the HC construct (n = 6 - 39) compared with those for the excised human cornea. The numbers denote the references where the values were extracted, as follows: (1-2, 5) Becker, 2006; (3) Woodward *et al.,* 2001; (4) Reichl *et al.,* 2005; Becker, 2006. When the error bar is not shown, the SD is smaller than the size of the symbol.

For the purpose of comparison between HC and excised rabbit cornea, six permeation markers, four ophthalmic drugs, two β-blockers were used and a correlation coefficient (R^2) of 0.85 was calculated (Figure 4-7). Interestingly, the P_{app} values for the hydrophilic permeation markers (FD-4, Na-FLU, mannitol and acyclovir) obtained with excised rabbit cornea (Majumdar *et al.,* 2008; Suresh *et al.,* 2010) and the HC construct were comparable. In contrast, larger differences between the P_{app} values reported and those measured in this work were found for urea and metoprolol. The urea P_{app} value for the HC construct turned out to be five-fold higher than the value reported by Klyce (Klyce, 1975), whereas, for metoprolol, it was about two-fold lower than the mean value obtained from the literature (Huang *et al.,* 1983; Xiang *et al.,* 2009). These discrepancies may arise from differences in the corneal layers of excised rabbit cornea and those of *in vitro* model based on human cornea. For instance, the hydrophilic stroma of the rabbit cornea is remarkably thinner than the reconstructed stroma of the HC construct (Chan *et al.,* 1983; Ojeda *et al.,* 2001; Baydoun, 2008). Thus it can be expected that rabbit cornea displays a higher permeability to the lipophilic compounds than HC and human cornea. This can be readily observed from Figure 4-7 and Figure 4-8. Moreover, another factor that might contribute to the variability is the high degree of intraspecie-specific variations reported for rabbit (Wang *et al.,* 1991; Majumdar *et al.,* 2008; Suresh *et al.,* 2010; Adelli *et al.,* 2015). Furthermore, the correlation between the data available from the literature on excised human cornea with the data obtained with the HC constructs is shown in Figure 4-8. The R^2 of 0.99 indicates a good correlation between the permeability of the HC construct and that of native human cornea.

The comparison of the permeation data obtained with the HC construct and those collected from the literature for excised human and rabbit cornea demonstrates that the P_{app} values correlated well between the models as is also confirmed by the calculated R^2 values.
The current investigation also shows that the HC construct exhibits barrier properties similar to those of excised rabbit and human cornea with regard to hydrophilic substances such as FD-4, Na-FLU, mannitol and aciclovir. This indicates that the permeability of the HC construct is the same to that of excised rabbit and human corneal tissue regarding hydrophilic substances.
Regarding lipophilic substances such as dexamethasone, timolol and rhodamine B, the P_{app} values of the HC construct and excised rabbit cornea were almost similar. Nevertheless, the P_{app} values obtained with metoprolol and propranolol differed between the models. An explication for such discrepancy is the structural difference of the stromal cell layers, in particular with regard to the thickness, between both corneal models as well as the high interlaboratory variability of the P_{app} reported in the literature. Despite of these differences, it was noted that the P_{app} values obtained with these two lipophilic substances for the HC construct were lower than the data reported for excised rabbit cornea. This indicates that the HC construct possesses a lower permeability to lipophilic substances than rabbit cornea. In contrast, the comparison of the P_{app} values obtained with propranolol and timolol showed that the values of the HC construct and excised human cornea were identical. This indicates that the thickness of the hydrophilic stroma of the HC construct is similar to that of human cornea. Unfortunately, a P_{app} value for metoprolol has yet not been reported for excised human cornea due to which a comparison to the values achieved with the HC construct is not possible. Thus, the HC construct has an equal permeability to lipophilic substances than excised human cornea.

4.2.3. Summary of the comparative analysis of transcorneal drug absorption

This chapter deals with the investigation of the physicochemical properties of substances on the permeation through the HC construct. Furthermore, the evaluation of the grade of comparability between the barrier properties of the HC construct and that of isolated rabbit and human cornea is described.
Eighteen different substances covering a wide range of octanol-water partition coefficients and molecular weights were used to evaluate thoroughly the permeability properties of the construct. Subsequently, the P_{app} values obtained with the HC construct were compared with the values available in the literature on excised rabbit and human cornea in order to assess the capability of the HC construct to show similar levels of permeability as the aforementioned excised corneal tissue.

The log *P* - P_{app} relationship for the different substances tested with the HC construct indicated that the lipophilicity of the substances is an important variable, which determines the permeation properties through the corneal barrier.
With regard to the lipophilicity of substances, the P_{app} correlates with the log *P*. In addition, it was demonstrated that the permeability of the HC construct increased when the lipophilicity increase according to a sigmoidal relationship. It has been previously reported that the permeability of isolated rabbit cornea increases sigmoidally with the log P (Wang *et al.,* 1991; Suhonen *et al.,* 1991; Suhonen *et al.,* 1998).
Considering the barrier properties of the HC construct, it is predicted that hydrophilic substances cross the corneal epithelium through the pores formed by tight junctions (paracellular pathway). This means that this pathway also limits the diffusion transport of substances depending on their MWs. Nevertheless, this assumption is only applicable to substances with large MWs (Hämäläinen *et al.,* 1997). In contrast, the transcorneal permeation of small substances is not influenced by their MWs (Prausnitz and Noonan*,* 1998). This observation is in good agreement with the data obtained in the current work.

In summary, the log *P* - P_{app} and the MW - P_{app} correlations demonstrate that, like *ex vivo* corneal tissue, the permeability properties of the HC construct are rather influenced by the lipophilicity of small substances but not their MWs.

Eleven of the eighteen substances evaluated with the HC construct have previously been tested with excised rabbit cornea. Despite the fact that the HC construct used in this investigation originates from human tissue, the comparison of its permeability with that of excised rabbit cornea (information available from the literature) showed a relatively high degree of correlation (R^2 = 0.85) with regard to these eleven substances. Furthermore, the slope of the regression line was 1.29. This value indicates that the permeability of excised rabbit cornea is in general 30% higher than that of the HC construct.
The comparison of the permeability characteristics of both corneal models indicated that, for hydrophilic substances (e.g. FD-4, Na-FLU, mannitol and acyclovir), the permeability of the HC construct was similar to that of excised rabbit cornea, whereas the permeability observed for lipophilic substances (bimatoprost, dexamethasone and rhodamine B), the permeability of the HC construct was close to the values published for excised rabbit cornea, whereas for metoprolol, the permeability of the HC construct was two-fold higher than the value found for excised rabbit cornea. The thickness of rabbit cornea, in particular, that of the hydrophilic stroma, is smaller than that of human cornea and thus the different P_{app} values obtained for some lipophilic markers might be a consequence of the different elongated diffusion distances of both corneal models (Chastain*,* 2003; Hahne and Reichl*,* 2011). This indicates

that the HC construct has a lower permeability to lipophilic substances than excised rabbit cornea.

Five of the eighteen substances evaluated with the HC construct have previously been tested with excised human cornea. As expected based on the origin of the HC construct, the comparison of the P_{app} values obtained with the HC construct with the ones reported for excised human cornea resulted in a linear relationship ($R^2 = 0.99$) for all 5 substances. This correlation was stronger than the one obtained for rabbit excised cornea. In addition, the resulting slope of the regression line was 0.94. The value suggests that the permeability of the HC construct is nearly equivalent to that of excised human cornea. However, the correlation is only based on five substances meaning a limited significance. Thus, further comparative permeation studies on ophthalmic drugs and model substances need to be conducted on excised human tissue in order to confirm the assumption mentioned above.
In short, the findings obtained within the scope of the current study suggest that the barrier properties of the HC construct are comparable to those of *ex vivo* human and rabbit cornea and that its development is sufficiently advanced to be safely considered for entering into the next steps toward its validation. This includes the evaluation of formulation factors, i.e. the optimization of buffer compositions of the ophthalmic solutions that will be described in the next chapters.

4.3. Effect of formulation parameters and excipients on the barrier function of the HC construct

4.3.1. Effect of pH ranging from 4.5 to 8.0 on the permeability of the HC construct

The pH of ophthalmic solutions plays an important role for the chemical stability of active compounds, drug bioavailability, as well as the associated ocular comfort or adverse reactions (Small *et al.,* 1997a; Small *et al.,* 1997b; Ahuja *et al.,* 2006). In this chapter, the effect of different buffer solutions frequently used in ophthalmic preparations, such as citrate, acetate and borate buffer and buffer solutions used in the cell cultivation such as PBS, HBSS and KRB (at different pH) were evaluated using the HC model based on the TEER values and the permeation of two hydrophilic permeation markers (Na-FLU and ^{14}C-mannitol, hereafter referred to as mannitol). These measured values were compared with those of KRB at pH 7.4, which served as control.

4.3.1.1 Influence of buffers: citrate pH 4.5, acetate pH 5.5 and borate pH 8.0

The effect of three ophthalmic buffer solutions (citrate buffer at pH 4.5, acetate buffer at pH 5.5 and borate buffer at pH 8.0) on the HC construct was evaluated. These solutions contained either the hydrophilic substance Na-FLU or mannitol, which served as transport solutions. The effect was evaluated by measuring the TEER (Figure 4-9) in KGM culture medium (black bars) as starting point. The TEER values achieved with each of the buffer solution were then measured after a preincubation time of 60 min before starting permeation experiments (gray bars) and at the end of the experiments after 390 min (pallid gray bars).

The Figure 4-9 clearly shows changes in the TEER values overtime caused by the different buffer solutions applied to the HC construct. However, only the citrate (pH 4.5) and borate buffer (pH 8.0) containing 10 µg·mL^{-1} of Na-FLU significantly affected the TEER values of the HC construct in comparison to KRB pH 7.4; the citrate buffer after 60 min and 390 min (end of the experiments) and the borate buffer only after 390 min. With respect to the effect of the buffer solutions containing 1.0 µCi·mL^{-1} of mannitol, the citrate buffer led to a slight decrease in the TEER values by 14% and the acetate buffer reduced the TEER values by 22% after 60 min of incubation. Both buffer solutions steadily reduced the TEER values of the HC construct to approximately 60% at the end of the experiments. Nevertheless, the observed reduction in the TEER values was not statistically significant when they were compared to the values achieved following the treatment with KRB at pH 7.4 (control solution).

The alterations caused by the citrate buffer at pH 4.5 containing Na-FLU can be explained by the fact that this buffer possibly leads to a depletion of the calcium ions and consequently a reduction in the TEER of the HC construct. This theory is supported by the fact that citrate buffer is a chelating agent (Akers, 2010) and calcium ions are required for the normal

functioning of the epithelial barrier membrane, which need an unspecified amount of calcium ions (Kaur and Smitha*,* 2002; Zheng and Cantley*,* 2007). The low pH value of the citrate buffer solution could be another reason for a possible damage caused in the superficial epithelial cells of the HC construct resulting in a decrease of barrier function. Therefore, the effect produced by the citrate buffer at pH 4.5 containing Na-FLU could be associated with its acidic pH and its capacity to form complexes with calcium.

Interestingly, the citrate buffer at pH 4.5 containing mannitol did not significantly affect the TEER values of the HC construct. This suggests that the assumption that the TEER value reduction has been caused by the acidic pH of this buffer and the possible depletion of calcium in the cells is questionable. However, since it was noted that citrate buffer with mannitol also led to a steady decrease in the TEER values throughout the duration of the experiments, the chelating properties and low acidic pH of this buffer are still the most probable reasons of the TEER values reduction. The indication that the citrate buffer containing mannitol did not cause a significant reduction of the TEER values could be explained by the fact that some excipients such as mannitol could have a protective effect on the HC construct. This assumption is supported by an observation recently made by Mohamed *et al*., who noted that mannitol reduced the effect of preservatives, such as benzalkonium chloride, on the TEER values of the corneal epithelium of rabbits (Mohamed *et al.,* 2016). In addition, a similar protective effect of mannitol against cytotoxic influence of benzalkonium chloride was also observed with regard to an *in vitro* corneal model based on the HCE-T cell line, which has also been used in the current investigation (Nagai *et al.,* 2011).

In contrast, the borate buffer with 10 $\mu g \cdot mL^{-1}$ of Na-FLU produced a lower reduction of the TEER values. However, the effect of borate buffer on the corneal epithelium is controversially discussed in the literature. Some investigations suggested that this buffer produces alterations in the tight junctions while others did not report any morphological change of the corneal epithelium (Gorbet *et al.,* 2010; Lehmann *et al.,* 2010).

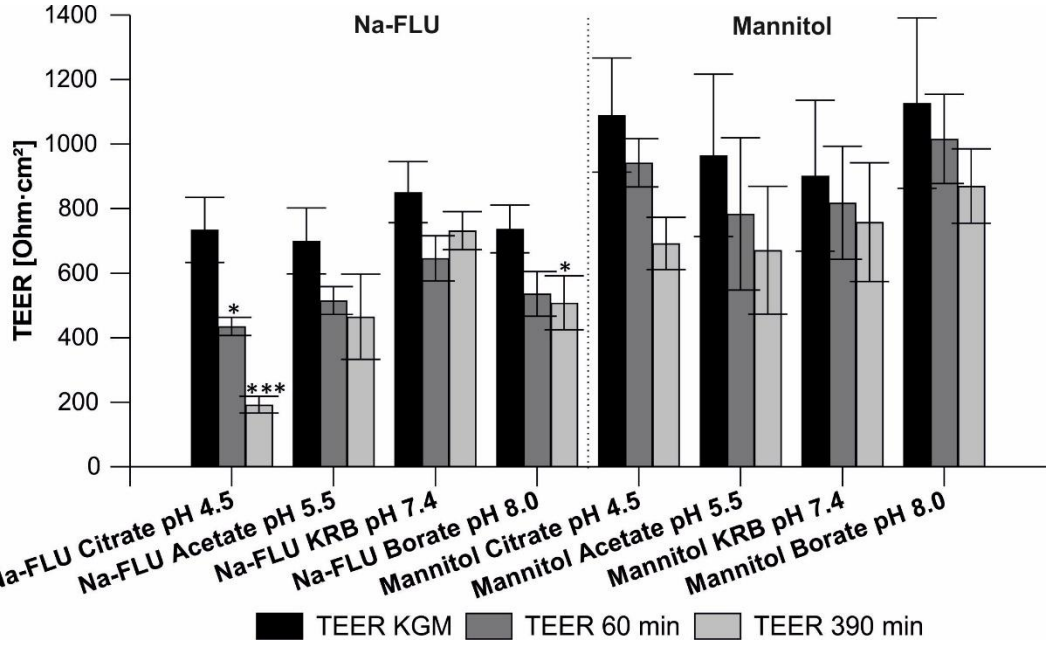

Figure 4-9: TEER values that the HC construct reached with citrate buffer at pH 4.5, acetate buffer at pH 5.5, KRB at pH 7.4 and borate buffer at pH 8.0 containing Na-FLU as well as mannitol. For the statistical analysis, the TEER values obtained with the buffer solutions were normalized to the mean value of the TEER in KGM (black bar) and compared to the data achieved with KRB at pH 7.4 after 60 min (gray bar) and 390 min (pallid gray bar), which served as control values. Each bar represents the mean ± SD of five to six independent experiments.

Besides the TEER measurements, the effect of citrate, acetate and borate buffer on the permeability of the HC construct was investigated using Na-FLU (Figure 4-10) and mannitol (Figure 4-11). Na-FLU is a hydrophilic permeation marker whose chemical form and log *P* is pH-dependent (Sjöback *et al.,* 1995), whereas mannitol is hydrophilic permeation marker, which is not influenced by changes in pH values. The P_{app} values of Na-FLU rose significantly under acid pH conditions (citrate and acetate buffer). A similar effect was observed when the pH of the solution was above 7.4 (borate buffer). Compared to the P_{app} values of KRB at pH 7.4, the P_{app} values of Na-FLU increased 37-fold for citrate, 4.9-fold for acetate and 5.8-fold for borate buffer. The strong enhancing effect observed for the citrate buffer on the permeability of the HC construct to Na-FLU was probably a consequence of the acid pH of the buffer solution, which made Na-FLU more lipophilic. Another reason, which could contribute to this effect, is the reduction of the TEER values of the HC construct (Figure 4-9).

The effect of the acetate and borate buffer on the permeability of the HC construct to Na-FLU could only be explained by a steady decrease in the TEER values and the fact that the acidic conditions increase the lipophilicity of Na-FLU. These results are consistent with reports on experiments with excised rabbit cornea showing that alterations in the pH of the buffer solutions affect the lipophilicity of pilocarpine prodrugs, which consequently changes the permeability of excised rabbit cornea to these substances (Suhonen *et al.,* 1998). In contrast,

the calculated P_{app} values of mannitol in citrate, acetate and borate buffers were equivalent to the P_{app} value of mannitol in KRB at pH 7.4. Thus, the P_{app} of mannitol was not affected by these buffer solutions. These results are also in good agreement with P_{app} values of mannitol observed for excised rabbit cornea when the pH of the buffer solution was decreased from 7.65 to 5.0 (Suhonen *et al.,* 1998).

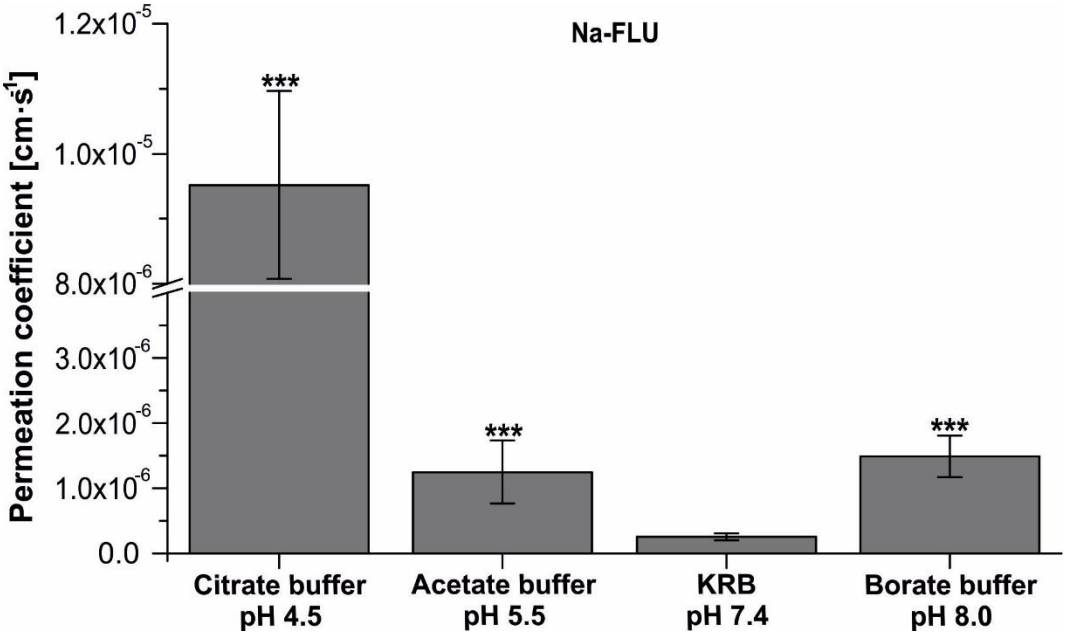

Figure 4-10: Influence of citrate buffer at pH 4.5, acetate buffer at pH 5.5 and borate buffer at pH 8.0 on the permeation of Na-FLU through the HC construct. For the statistical analysis, the P_{app} values were compared to the data obtained with KRB at pH 7.4 (control values). Each bar displays the mean ± SD of five to six independent experiments.

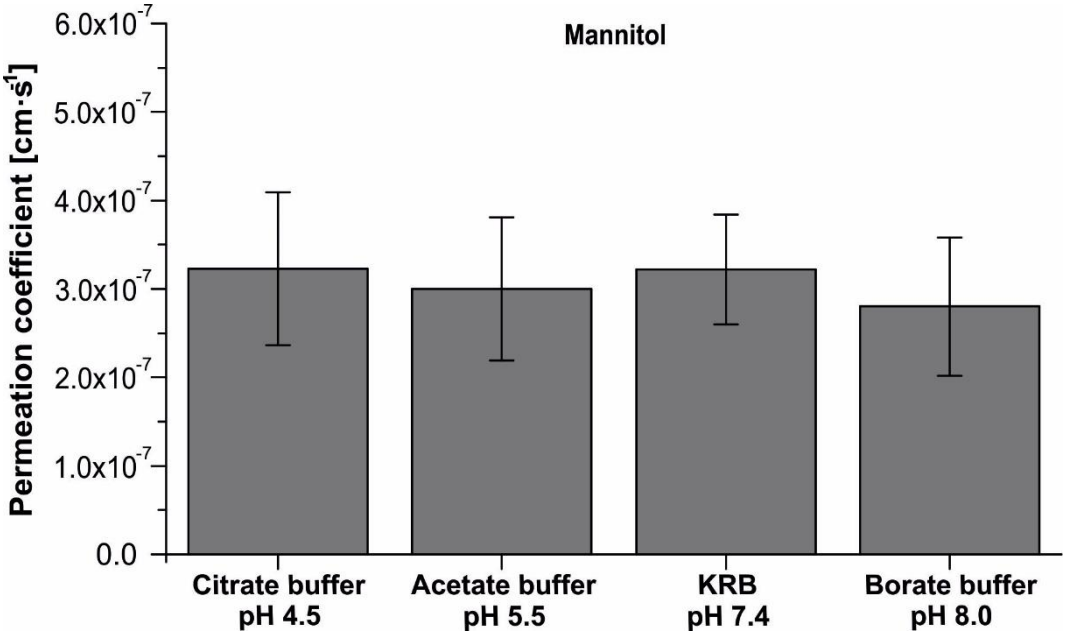

Figure 4-11: Influence of citrate buffer at pH 4.5, acetate buffer at pH 5.5 and borate buffer at pH 8.0 on permeation of mannitol through the HC construct. For the statistical analysis, the P_{app} values were compared to the data obtained with KRB at pH 7.4 (control values). Each bar displays the mean ± SD of five to six independent experiments.

4.3.1.2 Influence of KRB at pH 5.0, 6.0, 7.4 and 8.0

The buffer systems found in the human body are, for instance, the bicarbonate, phosphate and protein buffers. The Krebs-Ringer bicarbonate-buffer (KRB) is a mixed solution of bicarbonate and phosphate (see section 3.1.5), which can be supplemented with serum albumin or glucose. Thus, KRB is the solution, which is most commonly used within the scope of permeation studies since it provides a buffer capacity that is sufficiently strong (Becker *et al.,* 2007; Hakkarainen *et al.,* 2010; Hahne and Reichl*,* 2011). This part of the work focused on the investigation of the pH effect of KRB on the barrier function of the HC construct without altering the composition of the transport solution. The pH values investigated were 5.0, 6.0, 7.4 (control values) and 8.0 as is demonstrated from Figure 4-12 to Figure 4-14.

As described above, the black bars represent the TEER values of the HC construct in culture medium before the buffer solution was applied to apical side, the gray bars represent the TEER values after the preincubation time and the light gray bars show the TEER values after 390 min (end of the experiments). Neither KRB at pH 5.0, 6.0 and 8.0 with Na-FLU, nor KRB at the same pH containing mannitol significantly altered the TEER values of the HC construct after 60 min of incubation and at the end of the experiment. This indicated that the TEER values of the HC construct were not considerably altered by KRB at a pH between 5.0 and 8.0 (see Figure 4-12).

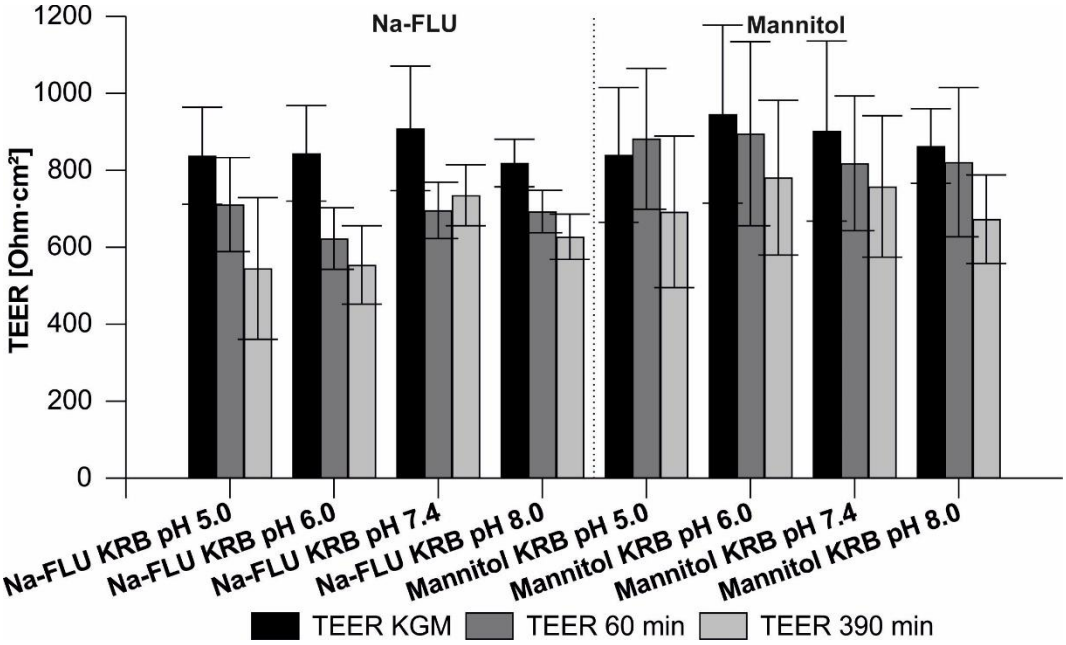

Figure 4-12: TEER values of the HC construct reached with KRB buffer at pH 5.0, 6.0, 7.4 and 8.0. For the statistical analysis, the TEER values obtained with the buffer solutions were normalized to the mean value of the TEER in KGM (black bar) and compared to the data achieved with KRB at pH 7.4 after 60 min (gray bar) and 390 min (pallid gray bar), which served as control values. Each bar represents the mean ± SD of five to six independent experiments.

The evaluation of the P_{app} indicated that KRB at pH 5.0 increased the P_{app} of Na-FLU about 12-fold. At pH 6.0, the P_{app} of Na-FLU was only 1.7-fold higher but not significantly different compared to KRB pH 7.4 (see Figure 4-13). Therefore, no significant alterations in the P_{app} values were observed. Moreover, when using KRB with a pH of 8.0, the P_{app} of Na-FLU was not modified and was almost equal to the P_{app} of Na-FLU in KRB at pH 7.4. Since no significant alterations were observed with regard to the TEER values of the HC construct (see Figure 4-12), the change in the permeation of Na-FLU can be attributed to the increased log *P* of Na-FLU when the pH of KRB was decreased from pH 7.4 to 6.0 and 5.0, respectively. The log *P* of Na-FLU in KRB at different pH was not determined in this study, but there is sufficient evidence, which demonstrates that the lipophilicity of ionizable substances increase in a dependent-manner when the pH of the buffer solution is modified (Cherng-Chyi and Lidgate, 1986; Suhonen *et al.,* 1998; Anand and Mitra, 2002; Ahuja *et al.,* 2006).

As already observed with regard to ophthalmic buffers, the permeation of mannitol was not affected by pH values in the same range. All P_{app} values determined were equivalent to the P_{app} value of mannitol in KRB at pH 7.4 (see Figure 4-14). This demonstrates that the corneal permeability of the HC construct is not changed by non-ionic compounds such as mannitol at pH values 5.0 to 8.0.

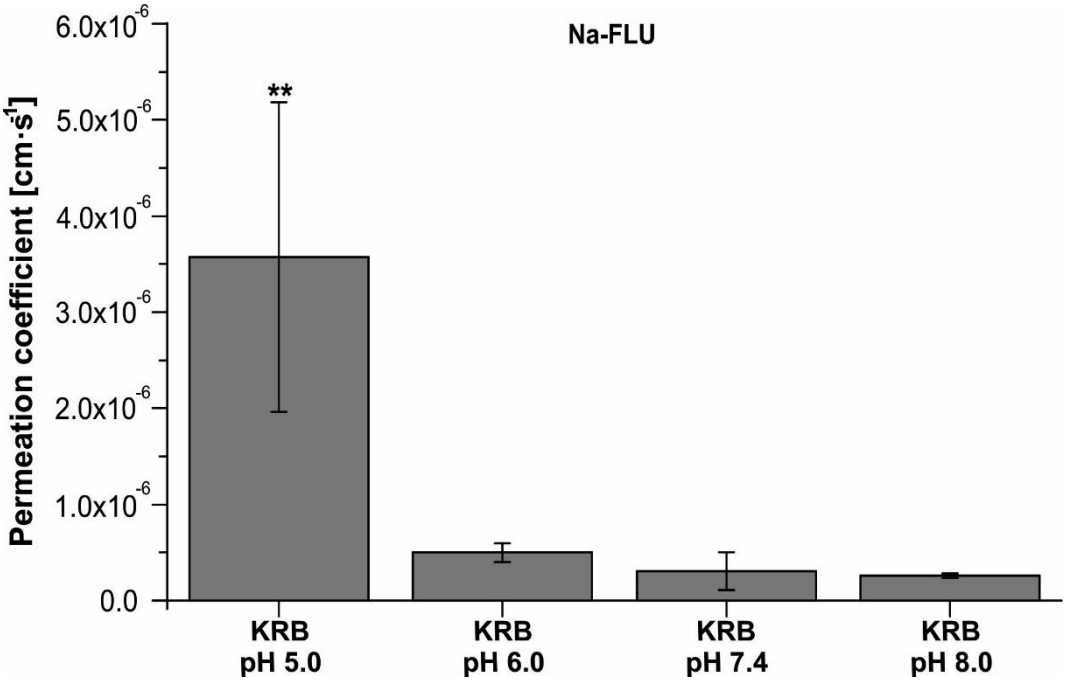

Figure 4-13: Influence of KRB at pH 5.0, 6.0 and 8.0 on the permeation of Na-FLU through the HC construct. For the statistical analysis, the P_{app} values obtained with KRB at pH 5.0, 6.0 and 8.0 were compared to the data obtained with KRB at pH 7.4. Each bar represents the mean ± SD of five to six independent experiments.

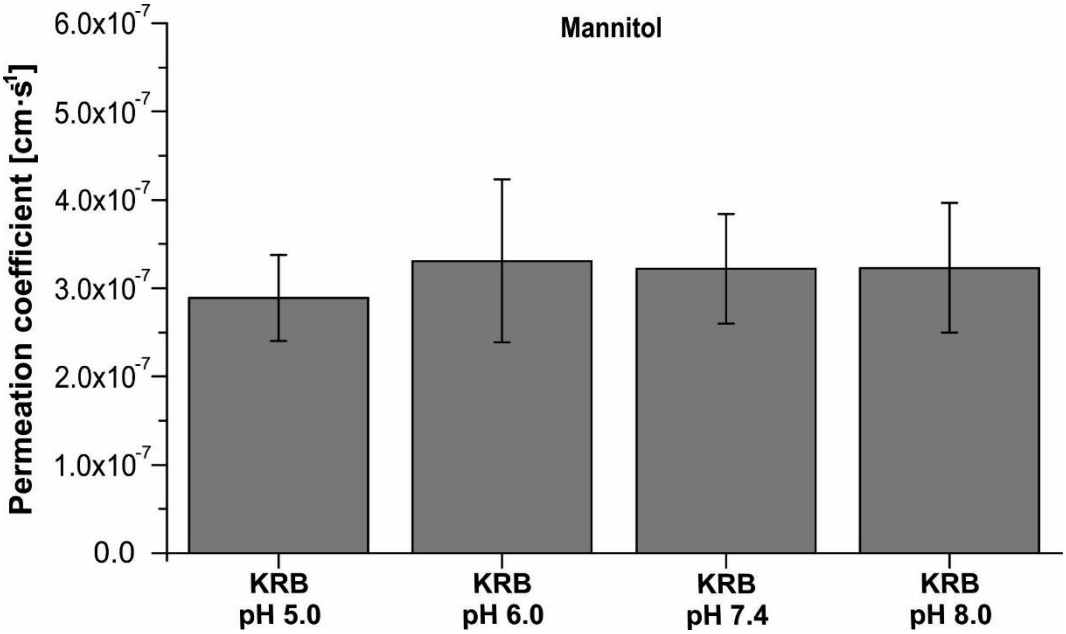

Figure 4-14: Influence of KRB at pH 5.0, 6.0 and 8.0 on the permeation of mannitol through the HC construct. For the statistical analysis, the P_{app} values obtained with KRB at pH 5.0, 6.0 and 8.0 were compared to the data obtained with KRB at pH 7.4. Each bar represents the mean ± SD of five to six independent experiments.

4.3.1.3 Influence of HBSS at pH 6.5, 7.4 and PBS at pH 7.4

The Hanks'-balanced salt solution (HBSS) has also been employed to conduct transport experiments. The main function of HBSS is to maintain the cell physiology and tonicity. This solution supplies cells with a balanced proportion of ions and energy.

The composition of HBSS permits to conduct permeability assays with different ionic concentrations compared to KRB and to investigate its influence on the barrier properties of the HC model. In addition, the effect of HBSS was evaluated at two different pH values, i.e. 6.5 and 7.4 (Figure 4-15 - Figure 4-17), using Na-FLU (10 µg·mL^{-1}) and mannitol (1.0 µCi·mL^{-1}), respectively.

The evaluation of the results indicated that HBSS at pH 6.5, 7.4 and PBS at pH 7.4 containing Na-FLU or mannitol did not have a negative effect on the TEER values after 60 min of incubation and at the end of the transport experiment compared with those obtained with KRB at pH 7.4, which served as control.

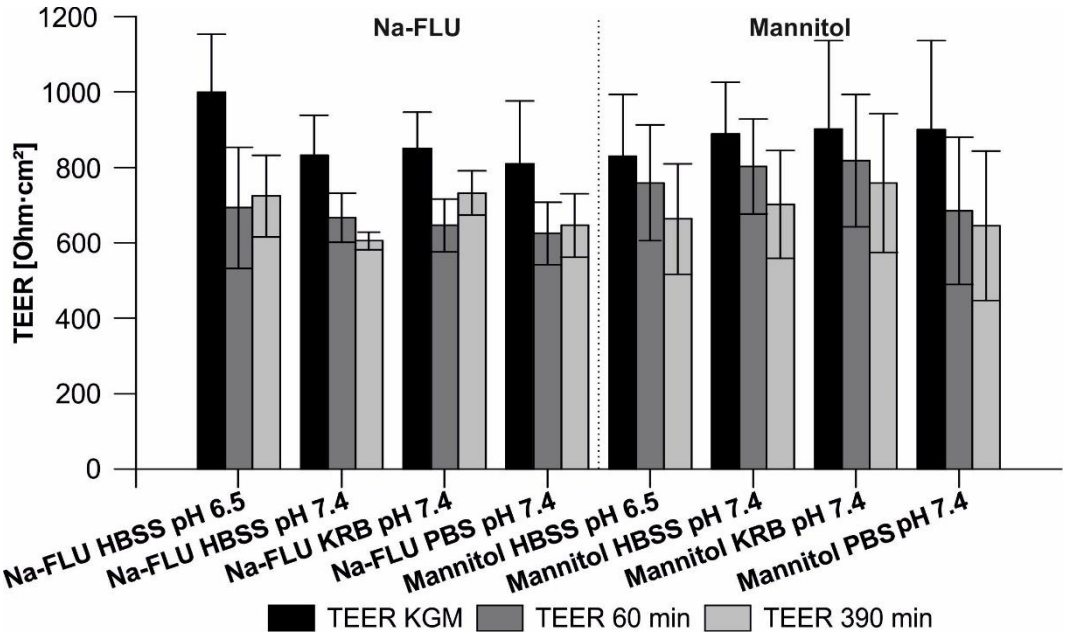

Figure 4-15: TEER values of the HC construct reached with HBSS at pH 6.5, 7.4, KRB at pH 7.4 and PBS at pH 7.4. For the statistical analysis, the TEER values obtained with the buffer solutions were normalized to the mean value of the TEER in KGM (black bar) and compared to the data achieved with KRB at pH 7.4 after 60 min (gray bar) and 390 min (pallid gray bar), which served as control values. Each bar represents the mean ± SD of five to six independent experiments.

In comparison of the effect of HBSS (pH 6.5 and 7.4) and PBS (pH 7.4) on the *in vitro* transcorneal permeation of Na-FLU, the transport rate of Na-FLU in HBSS at pH 7.4 was approximately two times higher than the one detected for the marker substance in KRB at pH 7.4 (Figure 4-16). Furthermore, the P_{app} of Na-FLU in HBSS at pH 6.5 was increased 6.86-fold compared to the mean P_{app} value of the reference value. The transport rate of Na-FLU in PBS was two-fold higher than the transport rate of Na-FLU in KRB at pH 7.4 (see Figure 4-16). Surprisingly, the measurements of the TEER values after 60 min and 390 min demonstrated the integrity of the *in vitro* corneal epithelium and no significant differences between HBSS, PBS and KRB.

The increase of the P_{app} values of Na-FLU in HBSS (pH 6.5) could be explained by the fact that the lipophilicity of Na-FLU increased when the pH of the HBSS was decreased from 7.4

to 6.5. The smaller but significantly effect of HBSS (pH7.4) and PBS (pH 7.4) could be due to different ionic strength of the buffer in comparison to KRB (pH 7.4), which increased the permeability of the HC construct to the cationic form of Na-FLU. The calculation of the ionic strength indicated that the ionic strength of HBSS and PBS at pH 7.4 was similar but significantly lower than that of KRB pH 7.4. The conclusion is based on the fact that diffusion potential experiments conducted by Rojanasakul and Robinson in 1989 using isolated rabbit. They previously demonstrated that the ionic strength of a buffer solution can influence the permeability of the corneal epithelium to ionic substances. They reported that the decrease of the ionic strength of the buffer solution resulted in an increase in the membrane selectivity to cations. In addition, the increase in the cation selectivity of the corneal epithelium led to an increase in the absolute permeability to cationic substances, and vice versa (Rojanasakul and Robinson, 1989). In contrast, the lipophilicity of mannitol (neutral molecule) was not affected by the pH of the buffer solutions. Therefore, the permeation of mannitol through the HC construct remained unchanged by HBSS at pH 6.5 compared to KRB at pH 7.4, as is represented in Figure 4-17. Moreover, within the scope of the current study, it was also noted that the ionic strength of HBSS, PBS at pH 7.4 did not influence the corneal permeability of the HC construct to mannitol in comparison to the data achieved with KRB at pH 7.4. Thus, it can be suggested that the ionic strength of buffer solutions has no effect on the membrane permselectivity to non-ionic substance such as mannitol.

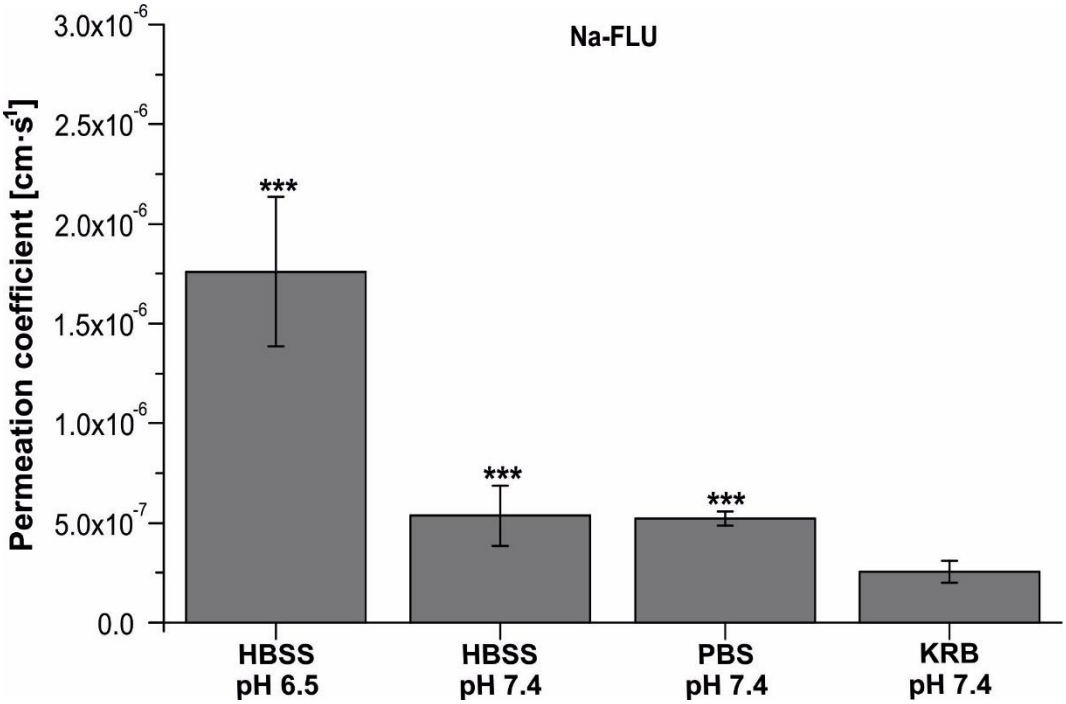

Figure 4-16: Influence of HBSS at pH 6.5, 7.4, KRB at pH 7.4 and PBS at pH 7.4 on the permeation of Na-FLU through the HC construct. For the statistical analysis, the P_{app} values obtained with HBSS at pH 6.5 and 7.4 and PBS at pH 7.4 were compared to the data obtained with KRB at pH 7.4 (control values). Each bar shows the mean ± SD of five to six independent experiments.

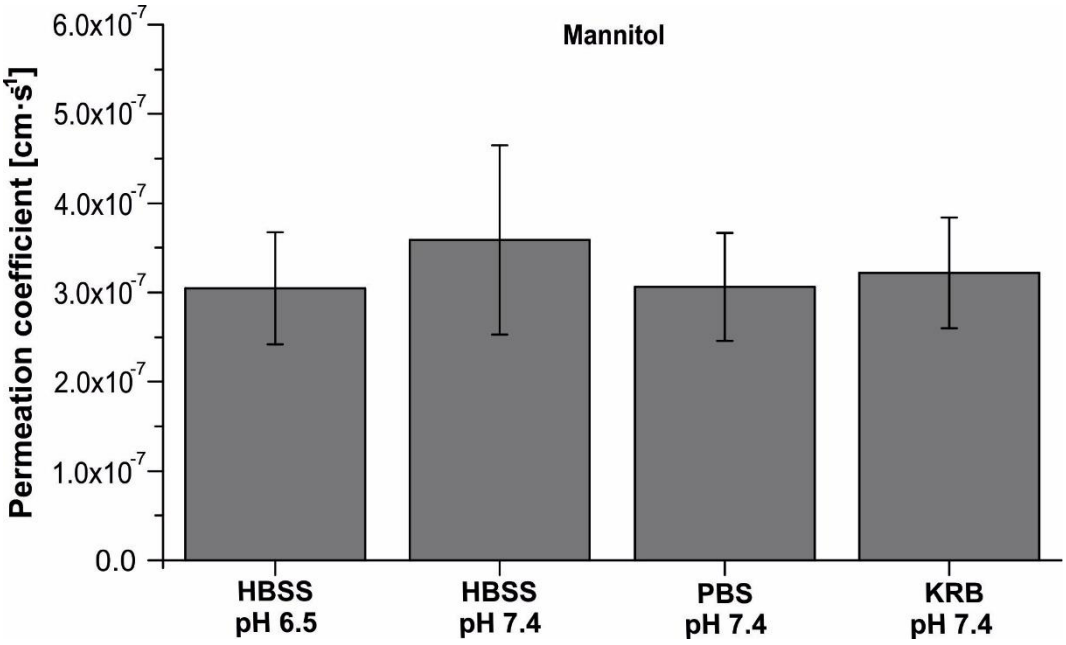

Figure 4-17: Influence of HBSS at pH 6.5, 7.4, KRB at pH 7.4 and PBS at pH 7.4 on the permeation of mannitol through the HC construct. For the statistical analysis, the P_{app} values obtained with HBSS at pH 6.5 and 7.4 and PBS at pH 7.4 were compared to the data obtained with KRB at pH 7.4(control values). Each bar shows the mean ± SD of five to six independent experiments.

4.3.2. Influence of osmolality

This section deals with the influence of non-isotonic solutions on the *in vitro* permeation of the hydrophilic substance Na-FLU through the HC construct. The osmolality of ophthalmic preparations is evaluated in the preclinical phase because hypotonic or hypertonic solutions produce ocular discomfort and its administration can be painful for the patient (Schrage *et al.,* 2004).

Within the scope of the present study, different osmolalities were obtained by modifying the concentrations of NaCl in the KRB (178, 252, 346 and 432 mOsmol·kg^{-1}) and used to assess the applicability of the present corneal model for preclinical evaluation and formulation design. The effect of the different osmolalities on the barrier properties of the HC construct and on the permeation of Na-FLU is illustrated in Figure 4-18 and Figure 4-19.

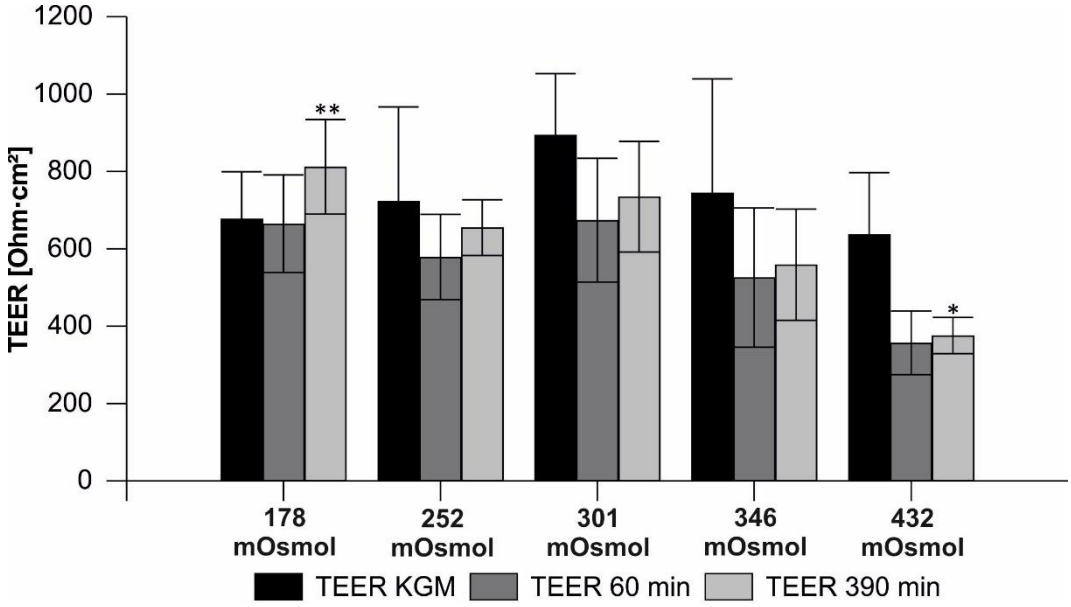

Figure 4-18: TEER values that the HC construct reached with different osmolalities ranging from 178 to 432 mOsmol. For the statistical analysis, the TEER values obtained with KRB with different osmolalities were normalized to the mean value of the TEER in KGM (black bar) and compared to the data achieved with KRB at 301 mOsmol (control values) after 60 min (gray bar) and 390 min (pallid gray bar) Each bar represents the mean ± SD of five to six independent experiments.

The TEER values were monitored before the donor solution was applied to the apical side (KGM medium, black bars). After 60 min as well as at the end of the permeation studies (390 min) the TEER values of the HC construct were measured again to compare the effect of the different osmolalities. After 60 min, no significant alterations of the TEER values were observed for the above-mentioned range of osmolalities. Nevertheless, the most hypoosmotic and hyperosmotic buffer had an impact on the TEER values of the HC construct, causing a significant increase or reduction of the TEER values after 390 min of transport experiments.

The P_{app} values of Na-FLU obtained with KRB (178, 252, 346 and 432 mOsmol·kg^{-1}) were compared with the P_{app} values of Na-FLU achieved with KRB at 301 mOsmol·kg^{-1} (see Figure 4-19). It can be noted that all P_{app} values of Na-FLU were almost equal in the range of the osmolality evaluated. Thus, the observed changes in the TEER values of the HC construct did not cause alterations of the Na-FLU transport. Nevertheless, it has been reported that the deviation of osmolalities from their physiological conditions can lead to alterations in the transport of substances through the corneal epithelium as demonstrated for pilocarpine. The transcorneal permeation of pilocarpine was decreased 1.28-fold when the osmolality of the vehicle was 600 mOsmol·kg^{-1}. In contrast, pilocarpine's transcorneal permeation increased 1.29-fold when the osmolality was 80 mOsmol·kg^{-1} (Scholz *et al.,* 2002). For the HC construct only a modest increase in the permeation of Na-FLU was observed when the osmolality of KRB was increased to 432 mOsmol·kg^{-1}, which can be associated with a slight reduction in the TEER values observed during the course of the

permeation experiments. Nevertheless, KRB at 432 mOsmol·kg^{-1} did not cause a significant change in the corneal permeability of the HC construct to Na-FLU compared with KRB 301 mOsmol·kg^{-1} control values

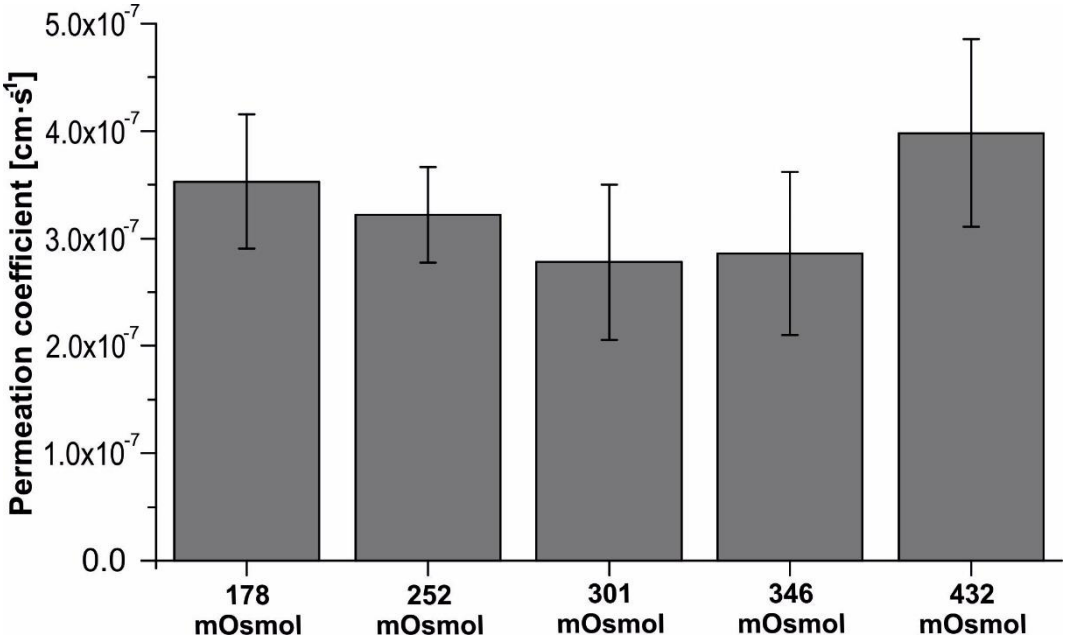

Figure 4-19: Influence of different osmolalities ranging from 178 to 432 mOsmol on the *in vitro* permeation of Na-FLU through the HC construct. For the statistical analysis, the P_{app} values obtained with KRB at 178, 252, 346 and 432 mOsmol were compared to the data obtained with KRB at 301 mOsmol (control values). Each bar displays the mean ± SD of five to six independent experiments.

4.3.3. Influence of EDTA and calcium

Ethylenediaminetetraacetic acid (EDTA) is a stabilizer and antioxidant agent used to maintain the stability of ophthalmic medications. Furthermore, this substance is known for its ability to enhance the properties of preservatives in ophthalmic products. EDTA is frequently found in eye drops in concentrations ranging from 0.01 to 0.1% (Furrer *et al.,* 2002). Epithelial barriers are formed by zona occludens and other associated proteins, which contribute to the formation of tight junctions. Among others, tight junctions control the paracellular transport of substances and stabilize cell junctions. The divalent ion calcium is known to assist in the formation of tight junctions (Westphal*,* 2009). Taking both into consideration, the effect of EDTA was evaluated on the HC construct for concentrations ranging from 0.005% to 0.1%, which is the maximum concentration used in ophthalmic preparations. The EDTA donor solutions were prepared in KRB with Na-FLU (10 µg·mL^{-1}) without calcium and magnesium. The effect of EDTA was compared with the values obtained for KRB (1.25 mM calcium with Na-FLU), hereinafter referred to as 0.0% EDTA, after 60 min and 390 min again, as illustrated in Figure 4-20.

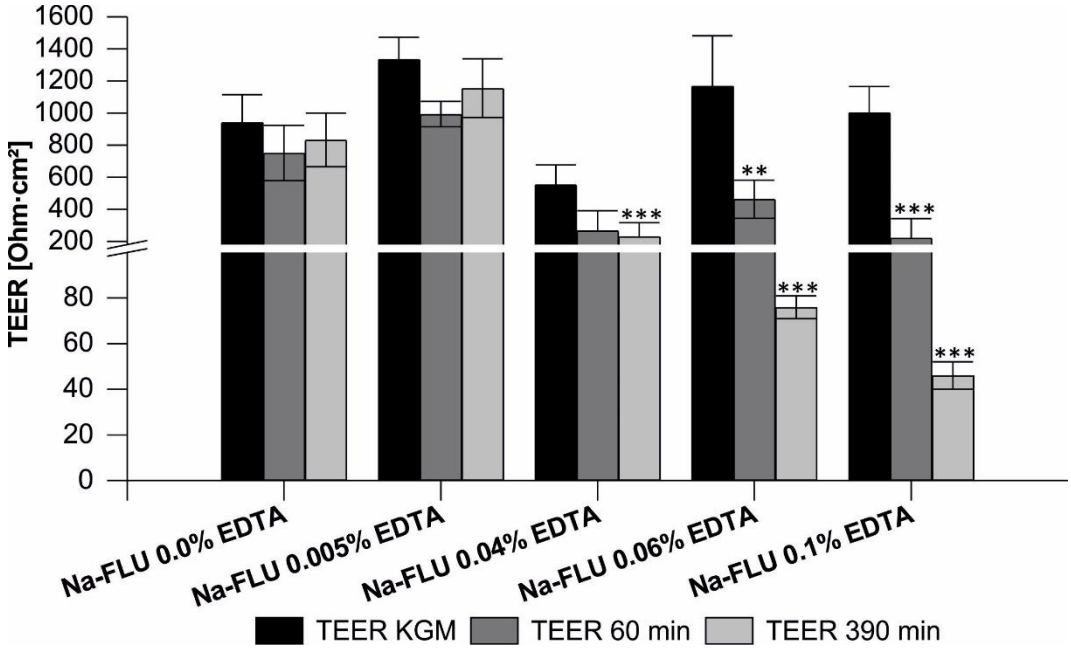

Figure 4-20: TEER values that the HC construct reached with different EDTA concentrations. For the statistical analysis, the TEER values obtained with different EDTA concentrations were normalized to the mean value of the TEER in KGM (black bar) and compared to the data achieved with KRB without EDTA (referred as 0.0% EDTA) after 60 min (gray bar) and 390 min (pallid gray bar), which served as control values. Each bar represents the mean ± SD of five to six independent experiments.

The effect of EDTA on the TEER values of the HC construct was concentration-dependent. It was noted that the TEER values of the HC construct were not affected when it was exposed to 0.005% EDTA, neither after 60 min nor at the end of the permeation experiments (390 min). In contrast, 0.04% EDTA reduced the TEER values by more than 50% of the initial TEER mean value (black bars) after 60 min of application. This effect was even stronger at concentrations of 0.06% and 0.1%. These values deteriorated even more at the end of transport experiments.

Similar results have been previously described for EDTA experiments using excised corneal tissue. In excised rabbit cornea 0.05% EDTA caused morphological alterations in the corneal epithelium after 180 min of incubation. Moreover, the treatment of rabbit cornea with 0.04% and 0.4% EDTA resulted in a decrease of the TEER values after the first 60 min of application (Rojanasakul *et al.,* 1990). These results are consistent with those obtained for the HC construct. Moreover, *in vitro* cytotoxicity studies using corneal epithelial cell cultures (human origin) indicated that the toxic effect of EDTA is dependent on the exposure time and the concentration used (Saarinen-Savolainen *et al.,* 1998).

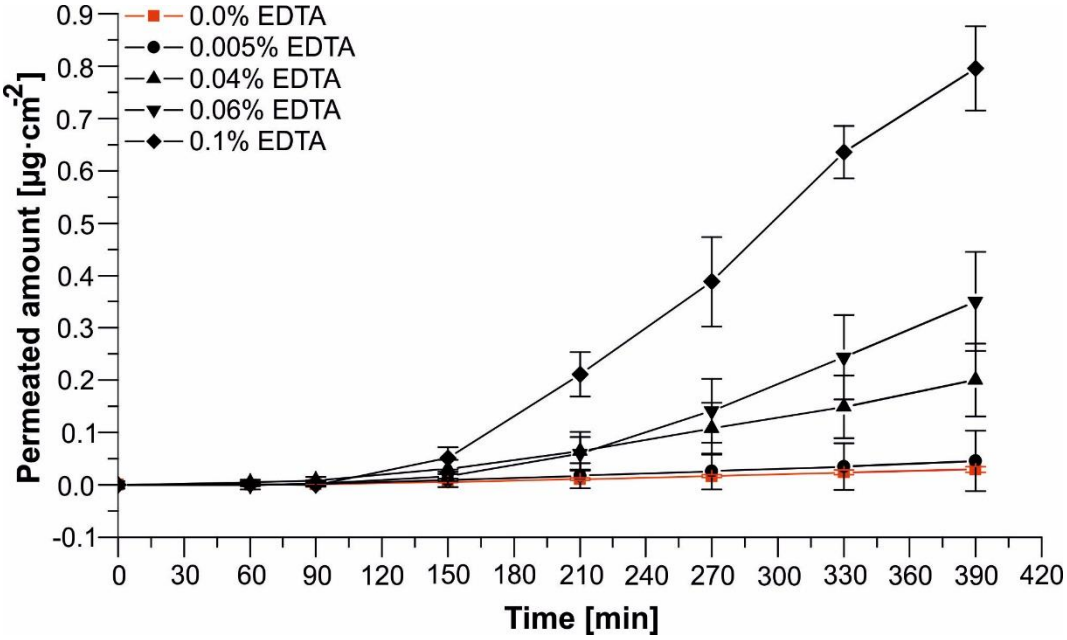

Figure 4-21: Permeated amount of Na-FLU with different concentrations of EDTA through the HC construct for a period of 390 min. For the statistical analysis, the calculated P_{app} values obtained with different concentrations of EDTA were compared to the data obtained with KRB without EDTA referred to as 0.0% EDTA (control values). Each point represents the mean ± SD of five to six independent experiments.

This disruption of the epithelial barrier correlates with an increase in the cumulative transport of Na-FLU by 27-fold for 0.1%, 12-fold for 0.06%, 7-fold for 0.04% and 2-fold for 0.005%, as shown in Figure 4-21. With regard to the calculated P_{app} through the HC construct, Na-FLU transport solutions with different concentrations of EDTA showed the same rank order of the cumulative transport described above for 0.1% > 0.06% > 0.04%.

The permeation of hydrophilic substances would preferably take place via the paracellular pathway. Thus, the increase in the permeated amount of Na-FLU, which was measured on the receptor side indicated that a loosening of the epithelial barrier occurred due to the depletion of calcium. The current data are in good agreement with the effect of EDTA previously reported by other investigation groups, which conducted experiments with isolated rabbit cornea. They showed that 0.5% EDTA increased the corneal permeability of isolated rabbit cornea to the hydrophilic substances, such as FD-4, about 3-fold (Nakamura *et al.,* 2007) and about 15-fold (Sasaki *et al.,* 1995b).

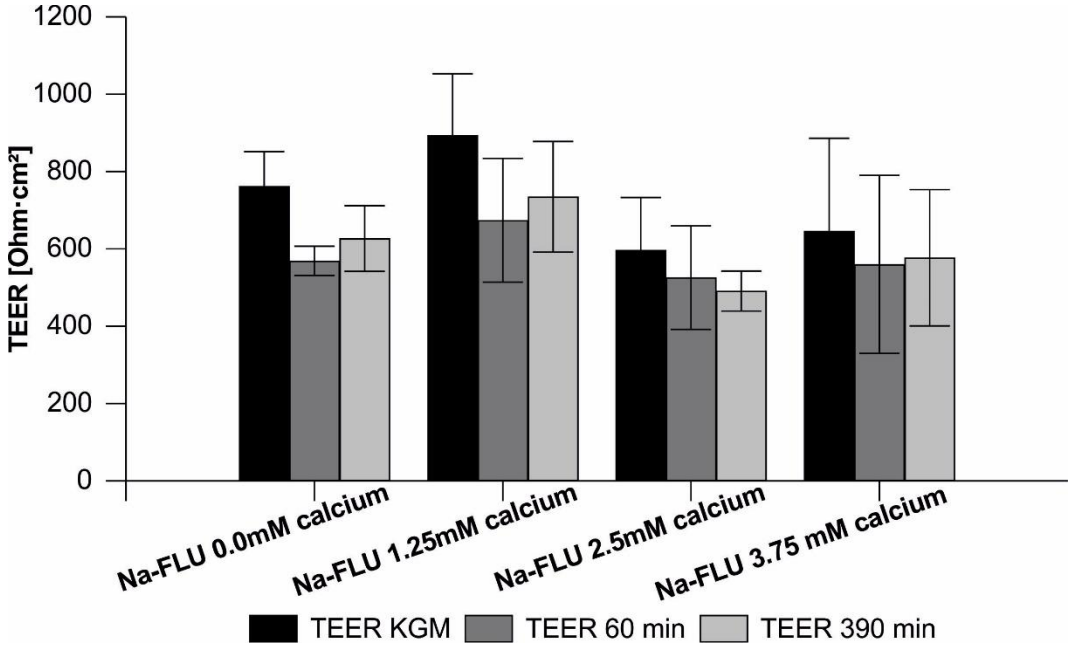

Figure 4-22: TEER values of the HC construct reached with KRB at 0.0, 1.25, 2.5 and 3.75 mM. For the statistical analysis, the TEER values obtained with KRB at different calcium concentrations were normalized to the mean value of the TEER in KGM (black bar) and compared to the data achieved with KRB at 1.25 mM (control values) after 60 min (gray bar) and 390 min (pallid gray bar). Each bar represents the mean ± SD of five to six independent experiments.

Calcium plays an important role regarding the tightness of epithelial barriers (Tang and Goodenough, 2003). Thus, calcium levels of 0.0, 1.25, 2.50 and 3.75 mM in the transport solution (KRB) were evaluated in the same way as done for EDTA. Concentrations higher than 3.75 mM calcium (e.g. 5 and 10 mM) led to the precipitation of KRB. The lack of calcium in the donor solution did not affect the TEER values of the HC construct during the course of permeability assay. The increasing of the calcium concentration to 1.25, 2.50 and 3.75 mM in the transport solution did neither cause a change in the TEER values nor an alteration in the permeated amounts of Na-FLU through the HC construct (see Figure 4-22 and Figure 4-23). The recorded TEER values were above 500 Ohm·cm². The fact that there was no change in the TEER values could be explained by the stromal equivalent of the HC construct being cultivated with 0.5 mM of $CaCl_2$, which is the concentration of calcium in KGM culture medium. In addition, the stromal equivalent provides the *in vitro* corneal epithelium with nutrients, which help to maintain the calcium homeostasis during permeability assays. This indicates that the solution 0.0 mM calcium was not completely calcium-free because the stromal equivalent was probably saturated with calcium.

Regarding calcium incubation of corneal tissue the effect on the corneal permeability has not yet been investigated within the scope of *ex vivo* studies with rabbit cornea. Thus, the data achieved with the HC construct cannot be compared to those obtained with *ex vivo* corneal tissue or with other *in vitro* corneal models.

Regarding calcium, the effect on the corneal permeability has not yet been investigated within the scope of *ex vivo* studies with rabbit cornea. Thus, the data achieved with the HC construct cannot be compared to those obtained with *ex vivo* corneal tissue or with other *in vitro* corneal models.

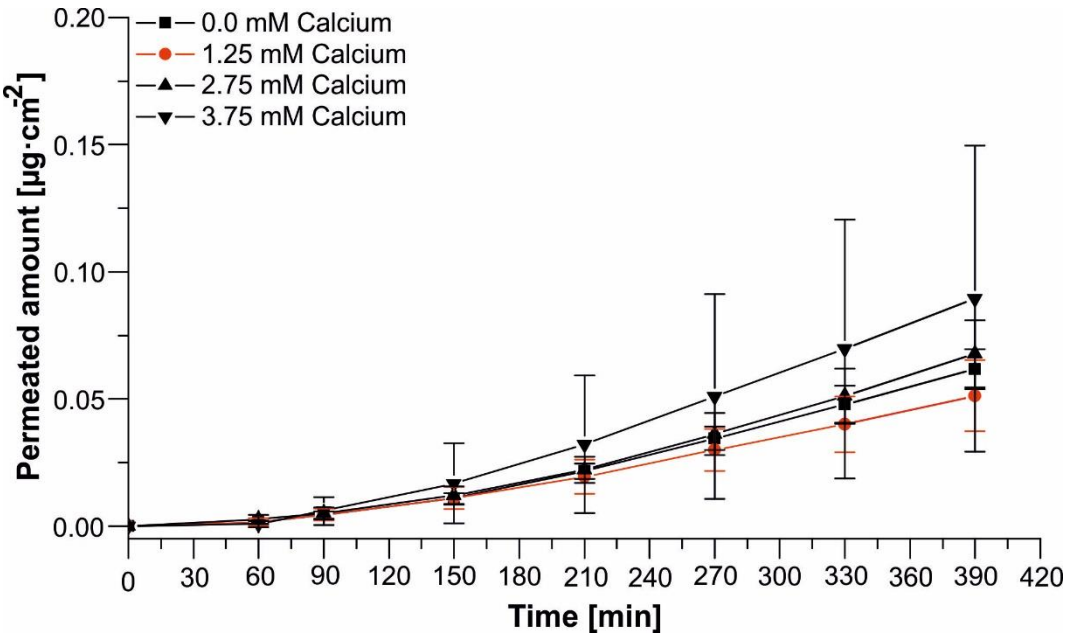

Figure 4-23: Permeated amount of Na-FLU with different concentrations of calcium through the HC construct for a period of 390 min. For the statistical analysis, the calculated P_{app} values with different concentrations of calcium were compared to the data obtained with 1.25 mM calcium (control values). Each point symbolizes the mean ± SD of five to six independent experiments.

4.3.4. Summary of the evaluation of the effect of formulation parameters and excipients on the barrier function of the HC construct

In this section, formulation parameters such as pH, osmolality and a number of ophthalmic adjuvants or excipients (i.e. EDTA and calcium), which are crucial to improve the stability and bioavailability of ocular drugs were investigated using the HC construct. The aim was to demonstrate HC's applicability in preclinical absorption studies during the phase of drug development. The changes in the corneal permeability induced by these formulation parameters and excipients were investigated by measuring the TEER values of the HC construct over time and by evaluating the permeability of the HC construct to Na-FLU and mannitol

4.3.4.1 Evaluation of buffer solutions in the range of pH 4.5 to 8.0

The results of the present investigation have shown the behavior of the barrier properties of the HC construct to different buffer solutions, covering a wide range of pH (4.5 to 8.0) in which ophthalmic solutions are commonly formulated. The buffer solutions evaluated in this study were the following: citrate buffer (pH 4.5), acetate buffer (pH 5.5), borate buffer (pH

8.0), HBSS (pH 6.5 and 7.4), PBS (pH 7.4) and KRB (pH 5.0, 6.0, 8.0) and KRB (pH 7.4); the values obtained with the latter were used as reference values.

The changes in the TEER values demonstrated that ophthalmic buffers with Na-FLU and mannitol, such as acetate buffer (pH 5.5), borate buffer (pH 8.0), HBSS at pH 6.5 and 7.4, PBS at pH 7.4 and KRB (pH 5.0, 6.0, 8.0) only cause minor alterations in the TEER values of the HC construct during the course of the experiments. Moreover, the application of citrate buffer (pH 4.5) with mannitol to the HC construct for the same time did not significantly affect the TEER values. In contrast, the incubation with the same citrate buffer (pH 4.5) containing Na-FLU resulted in a substantial reduction in the TEER values after 60 min, and an even higher decrease after 390 min.

Until today, there is no evidence, which confirms that the pH of the buffer solutions, in particular, citrate buffer at pH 4.5 causes any reduction in the TEER values obtained with isolated rabbit cornea or *in vitro* corneal models. Despite of the lack of evidence, it can be suggested that, on the one hand, the effect of citrate buffer with Na-FLU on the HC construct is due to the calcium chelator properties of this buffer or its low pH, which can affect the corneal integrity of the HC construct. On the other hand, these assumptions might be questionable considering that citrate buffer with mannitol did not cause any significant change in the TEER values. However, the results obtained with citrate buffer containing mannitol suggests that to some extent mannitol has a protective effect on the HC construct against citrate buffer at pH 4.5. This observation is supported by the toxicological studies previously conducted by Mohamed *et al*. and Nagai *et al*. with either isolated rabbit cornea or an *in vitro* corneal model based on the HCE-T line used in the current study. These investigations have shown that the presence of mannitol in ophthalmic agents can reduce the damaging effect of preservatives, such as benzalkonium chloride, on the superficial corneal epithelium of *ex vivo* rabbit cornea and the epithelium of the cultured corneal model, respectively (Mohamed *et al.,* 2016; Nagai *et al.,* 2011).

Furthermore, in the current study, minor changes were seen in the TEER values following the application of the other above-mentioned buffer solutions containing either Na-FLU or mannitol. This suggests that an optimal equimolar concentration of bicarbonate and phosphate in the buffer system similar to those found in the human body or lacrimal fluid provides the HC construct with the adequate conditions to protect itself against low acidic solutions.

In order to investigate how the pH of the buffer solution changes the permeability of the HC construct, two different hydrophilic permeation markers, Na-FLU, an ionizable substance, and mannitol, a non-ionizable compound, were used within the scope of the current investigation. The data obtained indicate that the permeability of the HC construct to Na-FLU can be increased without altering its TEER values when the pH of the buffer solution is

reduced from 8.0 to 5.0. This suggests that the lipophilicity of Na-FLU increases when the pH of the buffer solution is decreased. In addition, citrate buffer at pH 4.5 also led to both an increase in the corneal permeability to Na-FLU and a significant decrease in TEER values of the HC construct, which correlated with the highest P_{app} value achieved with buffer solutions containing Na-FLU.

Except for citrate buffer containing Na-FLU, the current findings are in line with permeability experiments previously conducted by other groups with *ex vivo* animal tissue. These experiments demonstrated that the corneal permeability of isolated rabbit cornea to ionizable hydrophilic substances, such as ketorolac, pilocarpine, diclofenac, amino acids ester prodrugs (e.g. L-Valyl ester of aciclovir), is increased when the pH of the buffer solution was decreased from 7.65 to 5.0 (Cherng-Chyi and Lidgate*,* 1986; Suhonen *et al.,* 1998; Anand and Mitra*,* 2002; Ahuja *et al.,* 2006). They demonstrated that the lipophilic characteristics of these substances changed when the pH of the buffer solutions were altered, which however did not affect the integrity of the corneal barrier. Consequently, it can be concluded that an increasing of the lipophilicity of hydrophilic substances by changing the pH of the buffer solutions results in an increase of their permeation rates through the corneal epithelium, which is the major barrier to transport of drugs through the cornea.

In addition, in the present study, it was seen that PBS and HBSS at pH 7.4 increased the corneal permeability of the HC construct to Na FLU compared to that observed with KRB at pH 7.4. Thus, the change in the corneal permeability to Na-FLU is a result of the different ionic strength of the buffer solutions. Moreover, the calculation of the ionic strength of these solutions indicated that the ionic strength of PBS and HBSS is similar but it is significantly lower than that of KRB. Thus, the data indicates that a lower ionic strength of the buffer solution could lead to an increase in the corneal permeability of the HC construct to Na-FLU considering that the predominant form of Na-FLU at neutral pH is the cation form as has been reported by Doughty in 2010. Therefore, these results are in agreement with the findings of Rojanasakul and Robinson who have demonstrated that a decrease in the ionic strength of the bath solution leads to an increase in the membrane permeability of isolated rabbit cornea to cationic substances, and vice versa (Rojanasakul and Robinson*,* 1989).

Furthermore, within the scope of the present study, it was observed that none of the evaluated buffer solutions with a pH in the range from 4.5 to 8.0 changed the corneal permeability of the HC construct to mannitol. Several previously performed permeability studies with *ex vivo* rabbit tissue also demonstrated that the corneal permeability of isolated rabbit cornea to non-ionizable hydrophilic permeation markers, such as mannitol or glycerol, was not significantly affected when the pH of the buffer solution was decreased from 7.65 to 5.0 since neither the lipophilicity of mannitol nor the corneal integrity was altered (Suhonen *et al.,* 1998; Fu and Lidgate*,* 1986; Ahuja *et al.,* 2006). In addition, there is no evidence

indicates that the ionic strength of a transport solution can alter the corneal permeability to non-ionizable hydrophilic substance such as mannitol.
Thus, the results obtained in the present study are in good agreement with the data collected from the literature with regard to isolated rabbit cornea.

In conclusion, the above-mentioned findings demonstrate that buffer solutions at pH values ranging from 5.0 to 8.0 are relatively harmless to the superficial epithelial cells of the HC construct since minor modifications have been noted with regard to the TEER values. In addition, the pH of the buffer solution can change the permeability of the HC construct to ionizable permeation markers (e.g. Na-FLU) but not to non-ionizable hydrophilic permeation markers (e.g. mannitol), which has also been previously reported for the isolated rabbit cornea. These findings support the conclusion that the HC construct is an *in vitro* corneal model, which can be used in the development of new ophthalmic solutions.

4.3.4.2 Influence of osmolality

This part of the current study was to investigate the effect of non-isotonic solutions on the corneal integrity of the HC construct and also to assess whether these solutions can produce an increase of the corneal permeability to the hydrophilic permeation marker Na-FLU. The effect of the osmolality was evaluated using KRB containing Na-FLU in the range of 178 to 432 mOsmol·kg^{-1}. The data obtained with KRB containing Na-FLU at 301 mOsmol·kg^{-1} served as control values.
The findings of the current study with the HC construct demonstrated that only KRB at 178 mOsmol·kg^{-1} led to an increase in the TEER values at the end of the transport experiments. In contrast, when the HC construct was exposed to KRB at 432 mOsmol·kg^{-1}, the TEER values significantly decreased after 390 min. Until today, there is no evidence, which confirms that the exposure of isolated rabbit cornea to non-isotonic solutions results in changes in the TEER values.
The analysis of the permeation rates of Na-FLU with the different osmolalities demonstrated that none of the non-isotonic solutions has been able to change the corneal permeability of the HC construct to Na-FLU. Previously conducted permeability studies with rabbit corneal epithelial cells demonstrated that non-isotonic solution at 80 mOsmol·kg^{-1} and 600 mOsmol·kg^{-1} changed the corneal permeability of the *in vitro* corneal model used (Scholz *et al.,* 2002). However, these osmolalities were out of the scope of the current study since no ophthalmic medication formulated with the mentioned osmolalities was found.
In conclusion, the application of non-isotonic ophthalmic solutions causes lacrimation, which accelerates the drug elimination process and consequently reduces the ocular bioavailability (Urtti*,* 2006). These factors play an important role *in vivo*. However, the *in vitro* HC construct

is not able to mimic these factors, which may be the reason why non-isotonic solutions have no effect on the corneal permeability of the HC construct.

4.3.4.3 Influence of EDTA and calcium

Ethylenediaminetetraacetic acid (EDTA) is a calcium chelating agent, which is frequently used in ophthalmic medications to improve the antimicrobial action of preservatives (Furrer *et al.,* 2002; Heydari *et al.,* 2013). Nevertheless, it has been reported that EDTA can affect the corneal integrity and change the corneal permeability to drugs (Grass *et al.,* 1985). Thus, one of the aims of the present study was to evaluate the effect of EDTA on the barrier properties of the HC construct. For this reason, EDTA concentrations ranging from 0.005% to 0.1% were dissolved in KRB containing Na-FLU (without calcium). The data obtained with KRB containing Na-FLU (without EDTA) served as reference values and the solution was defined as 0.0% EDTA. Furthermore, this KRB solution also contained 1.25 mM calcium, which is the extracellular concentration in mammalian cells.

In addition, the effect of calcium was also investigated at different concentrations within the scope of permeation studies, using KRB containing Na-FLU and calcium at concentrations ranging from 0.0, 1.25, 2.50 and 3.75 mM. Concentrations higher than 3.75 mM calcium (e.g. 5 and 10 mM) were not investigated since it was found that they led to the precipitation of KRB. The data obtained with KRB containing Na-FLU (1.25 mM calcium) served as reference values.

The findings of the current study indicate that the application of 0.005% EDTA to the HC construct has not caused any alteration in the TEER values during the entire course of the experiments. In contrast, 0.04% EDTA decreased the TEER values after 390 min. Moreover, when the HC construct was exposed to 0.06% and 0.1% EDTA, the reduction in the TEER values was even stronger after 60 min and 390 min than the reduction noted for 0.04% EDTA. This demonstrates that effect of EDTA on the HC construct is concentration and time-dependent. Moreover, it has also been demonstrated that the reduction in the TEER values correlated with a substantial increase in the corneal permeability of the HC construct to Na-FLU compared with the values obtained with KRB without EDTA.

The effect of EDTA noted with regard to the barrier function of the HC construct is in good agreement with that reported in the literature for isolated rabbit cornea. Rojanasakul *et al*. demonstrated that 0.05% EDTA damaged the superficial corneal epithelium after 180 min of application. In addition, they found that 0.05% and 0.5% EDTA led to a steady decrease in the TEER values, which started after the first 45 min of application and lasted until the end of the investigation (180 min) (Rojanasakul *et al.,* 1990). Furthermore, Saarinen-Savolainen *et al*. demonstrated that high concentrations of EDTA (e.g. 0.04%) significantly reduced the percentage of living cells of the cultured human corneal epithelial cells after 60 min of application (Saarinen-Savolainen *et al.,* 1998). Moreover, Nakamura and co-workers

demonstrated that 0.5% EDTA increased the corneal permeability of isolated rabbit cornea to hydrophilic permeation marker, such as FD-4, which correlated with a substantial reduction in the TEER values during the entire course of the experiments (Sasaki *et al.,* 1995b; Nakamura *et al.,* 2007). Moreover, it has been proposed that the effect of EDTA on the barrier function of the corneal epithelium is caused by calcium depletion on the cells that induces the disruption of the actin filaments and, as a consequence, a disruption of the tight junctions. The cytotoxic properties of EDTA have also been specified as a possible reason for its effect on the barrier function of the corneal epithelium (Rojanasakul and Robinson, 1991; Sasaki *et al.,* 1995b; Nakamura *et al.,* 2007).

Regarding the influence of the calcium concentrations during permeability assays, none of the tested calcium concentrations had an effect on the TEER values or the corneal permeability of the HC construct to Na-FLU. The present study is the first to investigate the effect of calcium during transport experiments, and, thus, there is no evidence available on isolated rabbit cornea to confirm the results obtained with the HC construct. In order to demonstrate that the results achieved within the scope of the present investigation are valid, the same experiments should be conducted with isolated rabbit cornea.

On the basis of the current results it can be concluded that EDTA affects the *in vitro* corneal integrity in a concentration and time-dependent manner and that it can also increase the corneal permeability of the HC construct to the hydrophilic permeation markers, such as Na-FLU, in a way that is similar to that previously reported for *ex vivo* experiment with isolated rabbit cornea. In addition, it was found that transport solutions without calcium or with calcium concentrations higher than 1.25 mM neither affect the TEER values nor the corneal permeability of the HC construct to Na-FLU.

Other kinds of excipients frequently found in ophthalmic medications are preservatives such as benzalkonium chloride, cetrimide and Purite®, among others. The effect of these substances on the barrier properties of the HC construct will be described in the next chapter.

4.4. Preservative effects on the HC construct

This section describes the influence of adjuvants, such as preservatives, which could besides their primary function also modify the *in vitro* epithelial barrier, facilitate or affect the permeation of drugs through the HC construct. Preservatives are commonly found in pharmaceutical preparations, such as ophthalmic solutions, and contact lens solutions. However, their use can cause adverse reactions that may lead to alterations of the ocular bioavailability. Thus, the impact of the most relevant opthalmic preservatives on the barrier properties of the HC construct and their influence in permeability assays were investigated.

4.4.1. Evaluation of preservatives using the HC construct

This investigation examined the influence of frequently used preservatives on the barrier properties of the HC construct. For this purpose, the changes of the TEER values were used again to determine the effects on the HC construct.

The effect of the preservatives was evaluated as described in section 3.2.8. In brief, for each preservative, 500 µL of a donor solution containing the respective preservative at different concentrations was added to the apical side of the HC construct. The, TEER values were then checked at different time points. The TEER values were evaluated to define an acceptance limit for the tolerance level of the HC construct for the preservatives. This acceptance limit was established in accordance with the quality criterion previously obtained during the transfer phase of the prevalidation of the HC construct (see section 4.1.1). Based on these requirements, the HC construct can tolerate a preservative concentration when both this concentration does not lead to a reduction of TEER values below 400 Ohm·cm² and the reduction of the TEER values does not exceed 50% compared to the initial TEER values.

The TEER values of preservative-free KRB served as negative control and 1.0% sodium dodecyl sulfate (SDS) solution served as positive control. The effect of the preservatives on the TEER of the HC construct is shown from Figure 4-24 to Figure 4-30.

4.4.1.1 Benzalkonium chloride

The preservative benzalkonium chloride (BAC) is a quaternary ammonium salt, which is active against fungi, gram-positive and gram-negative bacteria. BAC is found in ophthalmic preparations in concentrations from 0.004% to 0.02% (Ryan *et al.,* 2011). The effect of BAC on TEER was investigated for a concentration range from 0.0005% to 0.1% (see Figure 4-24).

For preservative-free KRB, which served as a negative control within the first 10 min a decrease of TEER values was detected, which is attributed to the experimental approach. After this initial decrease the TEER values stabilized in the range of 500-600 Ohm·cm² for the rest of the experiment. Furthermore, it can be seen that all concentrations of BAC

reduced the TEER values already after 10 min of application. The extent of this effect was dependent on the concentration of BAC. A stronger decrease of the TEER values than that caused by KRB was interpreted as a possible damage of the epithelial barrier of the HC construct. After 10 min, the TEER values observed for 0.0005% and 0.001% BAC increased steadily to above 450 Ohm·cm² until 60 min. Thereafter, the TEER values for BAC 0.0005% remained constant, while the TEER values for BAC 0.001% began to decrease slowly to about 321 Ohm·cm² until the end of the experiments. Higher BAC concentrations than 0.001% had a strong impact on the TEER values already after 10 min of application. These TEER values remained below 100 Ohm·cm² throughout the rest of the experiments.

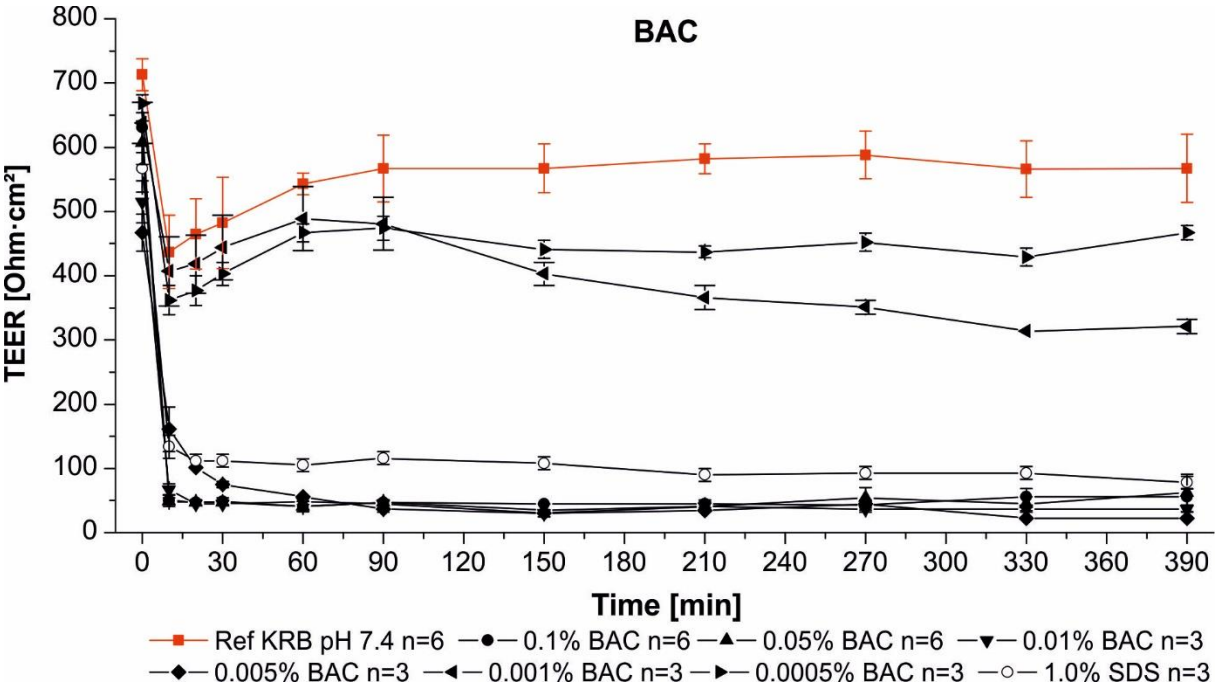

Figure 4-24: Mean ± SD of TEER values of the HC construct in function of time obtained after incubation with several concentrations of BAC compared to preservative-free KRB

Some previous studies have been described on the effect of BAC on corneal epithelium. *In vivo* studies with rabbits showed that 0.05% BAC led to a reduction of the TEER values from 600 Ohm·cm² to about 350 Ohm·cm² after only 5 s of application. In contrast, the TEER values of rabbit cornea treated with physiologic saline remained unchanged (control). Furthermore, the histopathological evaluation using electron microscopy showed that 0.05% BAC damaged the superficial corneal epithelial cells and the junctions between cells, which indicated the loss of tight junctions (Uematsu *et al.,* 2007). A similar *in vivo* study also disclosed that BAC concentrations higher than 0.002% caused a significant reduction of the TEER values after 1 min of administration, whereas concentrations lower than 0.002% did not significantly reduce the TEER values in comparison to the control values generated by the application of HBSS during the same time (Kusano *et al.,* 2010). Furthermore, an *ex vivo* study carried out with isolated rabbit cornea by Nakamura *et al.,* 2007 has demonstrated that

BAC concentrations of 0.002%, 0.01% and 0.05% concentrations are able to cause a reduction of the TEER values to about 45% (0.002%), 85% (0.01%) and 90% after 80 min of application. Within the scope of the further examination of the TEER values, the same group also noted that the epithelial resistances continued to decrease gradually over 160 min. Thus, these data are in good agreement with those obtained for the HC construct. In addition, Kusano *et al*. observed that the application of 0.005%, 0.01% and 0.02% BAC for 60 s reduced the percentage of living cells of *in vitro* cultivated rabbit corneal epithelial cells to about 30%, 70% and 80% in comparison to the control values (Kusano *et al.,* 2010). In contrast, concentrations lower than or equal to 0.002% neither reduced the percentage of living cells nor the TEER values (Kusano *et al.,* 2010). Furthermore, this group has been able to establish a linear correlation between TEER values and cell vitality. Moreover, in a series of *in vitro* experiments, Ayaki *et al*. reported cytotoxic effects for cultured human and rabbit corneal epithelial cells when these were exposed to ophthalmic solutions with BAC concentrations of 0.005%, 0.01% and 0.015% for 10 min, 20 min, 30 min, 60 min and 48 hours. They concluded that BAC had a toxic effect on the cultured cells that was dependent on the concentration and the exposure time (Ayaki *et al.,* 2008; Ayaki *et al.,* 2010).

The effect seen in the current investigation with regard to the different BAC concentrations on the HC construct is equivalent with those found for *in vivo* and isolated rabbit cornea. Furthermore, it can also be suggested that there is a strong correlation between the BAC concentration and the reduction in TEER values, which is probably due to the toxic effect of BAC on the reconstructed corneal epithelium as has also been observed within the scope of *in vitro* toxicological experiments. All these facts indicate that the HC construct has a level of sensitivity to BAC, which is comparable to that reported for rabbit cornea

4.4.1.2 Cetrimide

The preservative cetrimide (Ce) is also a quaternary ammonium salt like BAC. The difference between Ce and BAC and is that the latter is effective against gram-positive, gram-negative bacteria and has also wide spectrum against fungi. However, Ce is not active against all bacteria and fungi. The maximum concentration of Ce used in pharmaceutical eye drops is 0.005% (Furrer *et al.,* 2002; Bron *et al.,* 1998).

The effect of this preservative was investigated in the HC construct for a concentration of 0.0005% and 0.005% (see Figure 4-25). The evaluation of the TEER values indicated that the barrier function of the HC construct exceeded the limit of 400 Ohm·cm² following the application of 0.0005% and 0.005% Ce and remained unaffected throughout the course of the experiments.

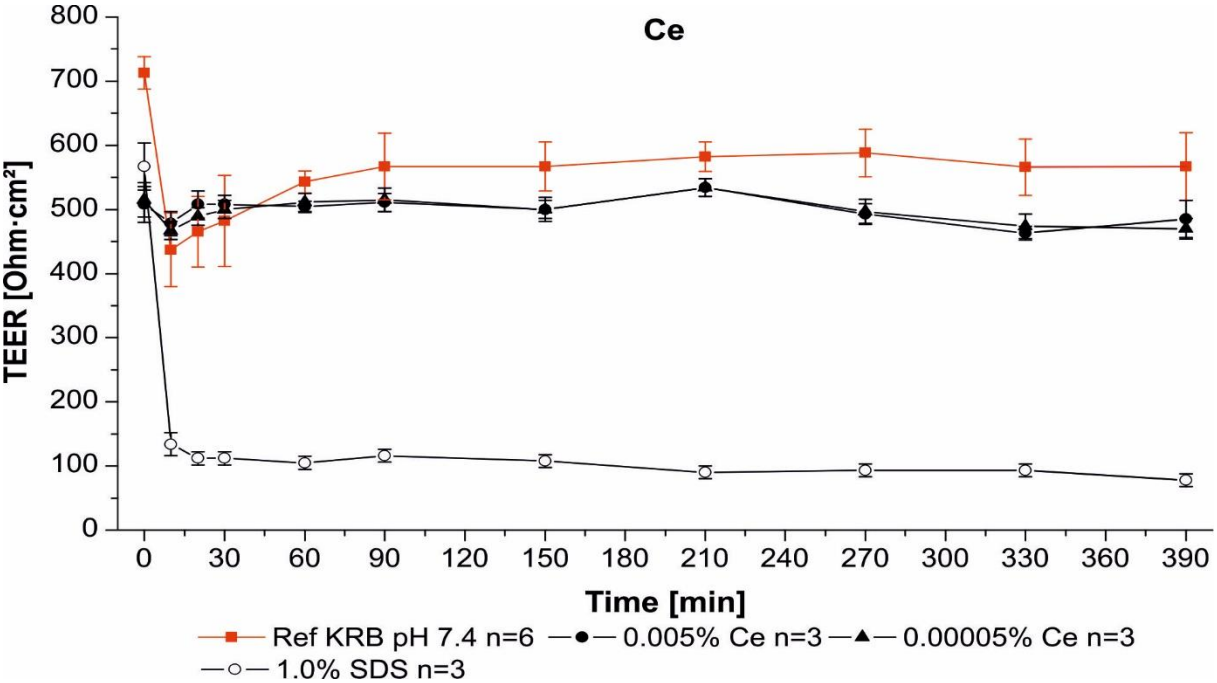

Figure 4-25: Mean ± SD of TEER values of the HC construct in function of time obtained after incubation with some concentrations of Ce compared to preservative-free KRB

Unfortunately, no TEER data are yet available in the literature for Ce using *in vivo, ex vivo* or *in vitro* corneal models. Hence, the results obtained for Ce with the HC construct cannot directly be compared with the aforementioned corneal models. However, several research groups have used microscopic analyses to evaluate the effect of ophthalmic solutions or excipients, taking into account the corneal epithelial cell morphology (Furrer *et al.,* 2000; Reichl *et al.,* 2005; Kusano *et al.,* 2010). Microscopic analyses are used to control the integrity of the superficial cells and the tight junctions between them. In this context confocal microscopy experiments on albino rabbits have demonstrated that 0.01% and 0.02% Ce instilled into the rabbit eyes, three drops daily for about three weeks, did not produce any morphological alteration in the corneal epithelium and stromal cell layers (Mahmoud, 2007; Mahmoud *et al.,* 2011). Furthermore, Matsuda *et al*. have also demonstrated that high Ce concentrations are toxic to the corneal epithelial cells. In their experiments, they observed that the application of 0.05%, 0.5% and 1.0% Ce for 30 min reduced the percentage of living cells of cultured rabbit corneal epithelial cells (3D corneal model) to about 60%, 10% and 5% respectively (Matsuda *et al.,* 2009).

The above-mentioned evidence revealed that 0.01% Ce does not significantly change the morphology of rabbit cornea suggesting no influence on the barrier properties of the corneal epithelium. These findings are consistent with the results obtained within the scope of the current investigation.

4.4.1.3 Methylparaben

The preservative methylparaben (MP) is a member of the parabens family. MP is an ester of p-hydroxybenzoic acid, which is effective against fungi but has only a limited activity against bacteria. This preservative is not frequently used in ophthalmic solutions. Nevertheless, it can be found in concentrations between 0.001% and 0.1% (Epstein *et al.,* 2009; Furrer *et al.,* 2002). This concentration range was evaluated with the HC construct (see Figure 4-26). It can be noted that all concentrations of MP altered the TEER values of the HC construct after the first 10 min of application again. In addition, it can be seen that MP concentrations of 0.001% and 0.05% caused roughly the same effect as preservative-free KRB after 20 min. In contrast, a stronger decrease of the TEER values was observed for the MP concentration of 0.1%. After 30 min of application, the TEER values for 0.001% MP were still above 450 Ohm·cm² and remained at this level until 390 min, whereas the TEER values for 0.05% MP began to decrease slightly to about 270 Ohm·cm² until the end of experiment. In contrast, 0.1% MP led to a gradual decrease in the TEER values from 250 Ohm·cm² to about 200 Ohm·cm² until 60 min of incubation. After this time they fell below 100 Ohm·cm² until 390 min of incubation.

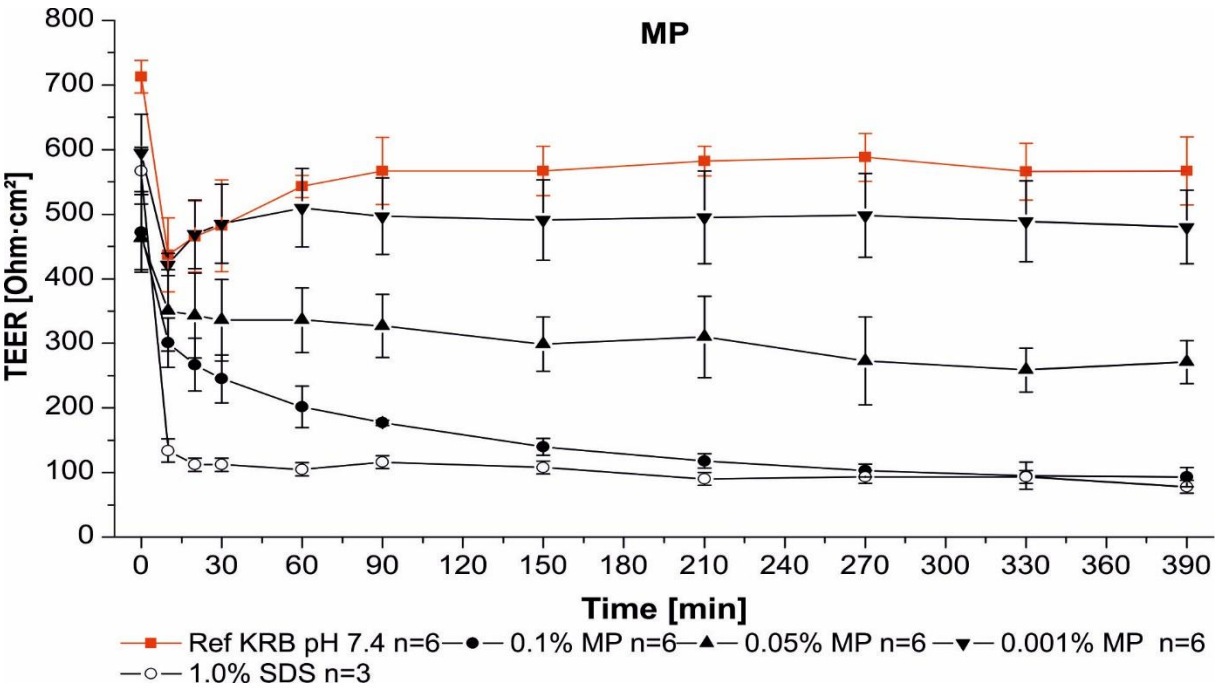

Figure 4-26: Mean ± SD of TEER values of the HC construct in function of time obtained after incubation with several concentrations of MP compared to preservative-free KRB

Unfortunately, the effect of MP on the TEER values measured on isolated human or rabbit cornea has not yet been reported to date. Thus, a comparison between the TEER values of the different corneal models with regard to the preservative MP is not available. Nevertheless, one research group used confocal microscopy to evaluate the effect of MP on

corneal epithelial cell morphology following its topical application. They conducted *in vivo* animal experiments with rats and demonstrated that a daily dose of 0.005% MP instilled into the eyes of the test animals for one month led to significant morphological alterations in the corneoconjuntival epithelium (Becquet *et al.,* 1998). In addition, further toxicological *in vitro* experiments have been conducted to evaluate the effect of MP on cultured human corneal epithelial cells. For instance, one *in vitro* study demonstrated that more than 50% of the cells survived after 60 min of incubation with 0.004% MP (Saarinen-Savolainen *et al.,* 1998). Another *in vitro* study described that the treatment of cultured human corneal epithelial cells with 0.001%, 0.05% and 0.1% MP for 60 min resulted in the death of approximately 36%, 67% and 77% of the cells (Epstein *et al.,* 2009). Altogether, these studies indicate that MP at concentrations ranging from 0.004% to 0.1% significantly changes the superficial corneal epithelium. Thus, the results of the current investigation are in good agreement with those effects seen in *in vivo* and *in vitro* experiments.

4.4.1.4 Thiomersal

The preservative thiomersal (Thio) is an organomercury substance and is used as bacteriostatic and fungistatic agent in ophthalmic solutions in concentrations from 0.001% to 0.02% (Furrer *et al.,* 2002); 0.0025% is the most frequently used concentration (Epstein *et al.,* 2009). The influence of Thio on the HC construct was investigated for a concentration range from 0.00005% to 0.02% (see Figure 4-27). The TEER values for Thio 0.00005%, 0.0005% and 0.005% indicated that these concentrations induced the same effect as preservative-free KRB during the first 60 min of exposure. After 60 min, the TEER values for 0.0005% and 0.005% began to exhibit a different trend. For KRB the TEER values increased slightly and remained constant, while for Thio 0.00005% a slight decrease in the TEER values from 550 Ohm·cm² to about 450 Ohm·cm² was observed until the end of the experiments. However, the TEER values for Thio 0.0005% and 0.005% constantly decreased until they reached a value of below 200 Ohm·cm² after 390 min of exposure.
With respect to the highest Thio concentration tested (0.02%), a stronger effect was observed during the first 60 min of incubation in which the TEER values decreased even more than the values for Thio 0.005%. A steady decrease of the TEER values was observed after this time. The TEER values fell to approximately 100 Ohm·cm² until the end of the experiments.

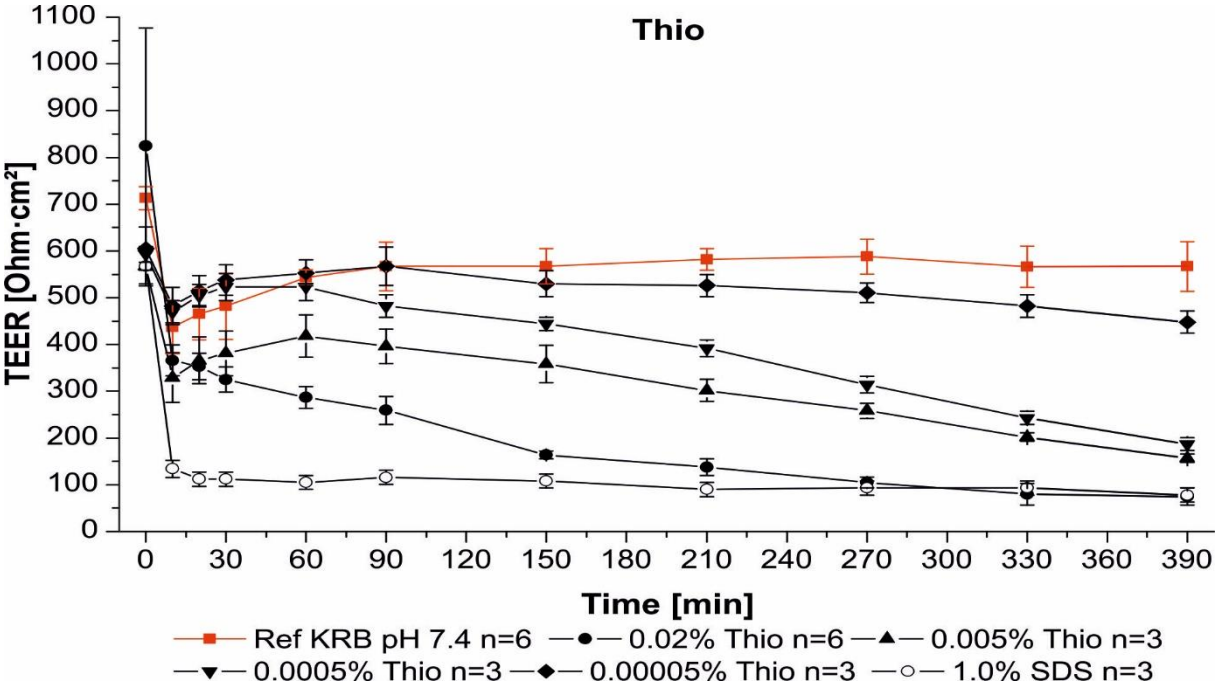

Figure 4-27: Mean ± SD of TEER values of the HC construct in function of time obtained after incubation with several concentrations of Thio compared to preservative-free KRB

The data obtained in the current investigation are in good agreement with those obtained by Burstein and Klyce in 1977. In their study they investigated the effect of Thio in the range from 0.00004% to 0.0004% on isolated rabbit cornea. For instance, 0.0004% Thio led to the detachment of approximately 50% of the superficial epithelial cells after 90 min of application. This change in the corneal epithelium correlated with a reduction of the transcorneal resistance. Furthermore, a decrease of the transcorneal resistance did not occur until 45 min of incubation, the point when the process of detachment of superficial epithelial cells started. At a concentration of 0.0001%, Thio affected to some extent the epithelial surface of the isolated rabbit cornea after 90 min of incubation. In contrast, 0.00004% Thio neither produced a morphological alteration of the superficial epithelial cell layers nor a change in the transcorneal resistance after 120 min of incubation (Burstein and Klyce, 1977). Moreover, it has been demonstrated in *in vitro* experiments that 0.0001%, 0.0005% and 0.01% Thio reduces the percentage of living human corneal epithelial cells to about 30%, 20% and 4% after 60 min of incubation (Epstein *et al.,* 2009). These results suggest that Thio can affect the integrity of the corneal epithelium in concentrations ranging from 0.0004% to 0.01%. Thus, the data reported for *ex vivo* rabbit cornea and *in vitro* corneal models are consistent with the data obtained with the HC construct.

4.4.1.5 Polyquaternium-1

The preservative polyquaternium-1 (Polyquad® - PQ-1) is a polymeric quaternary ammonium salt with antimicrobial activity. The effect of PQ-1 on the HC construct was evaluated at concentrations ranging from 0.0000005% to 0.0005% (see Figure 4-28).

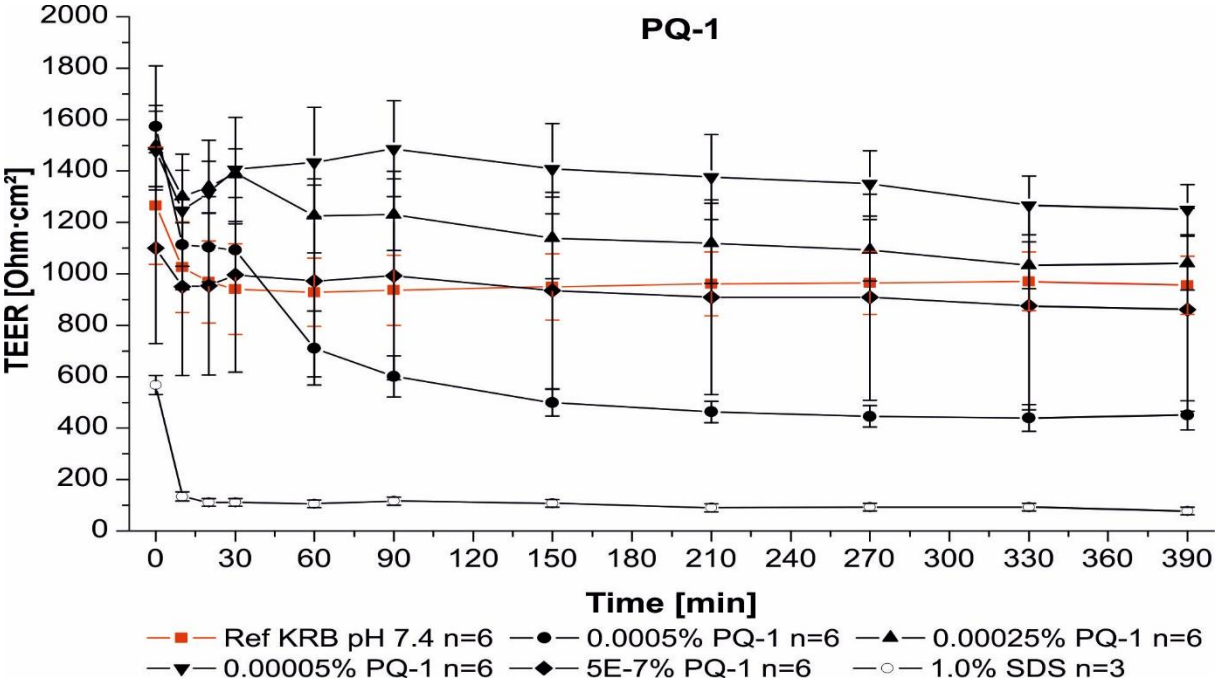

Figure 4-28: Mean ± SD of TEER values of the HC construct in function of time obtained after incubation with several concentrations of PQ-1 compared to preservative-free KRB

After 30 min of application all concentrations of PQ-1 affected the TEER to an extent, which is roughly comparable to preservative-free KRB. Thereafter, PQ-1 0.0000005% increased the TEER values until 90 min, whereas PQ-1 0.00025% slightly decreased the TEER values until 60 min. Afterwards, the TEER values for these concentrations of PQ-1 were relatively stable until the end of the assays. In contrast, for PQ-1 0.0005%, the TEER values were only stable until 30 min. A steady decrease in the TEER values was observed after this time. The values decreased to approximately 450 Ohm·cm² until the end of the experiments.

Although, in the current *in vitro* study, the maximum PQ-1 concentration evaluated was 0.0005%, the current results are congruent with those obtained *in vivo* by other research groups. Within the scope of an *in vivo* study described in 1991, the authors first exposed rabbit cornea to ruthenium red (a diagnostic substance that is used to detect the disruption of tight junctions) and then the rabbit cornea was treated with 0.001% PQ-1 for 90 min and 180 min. Transmission electron microscopy was used to examine the isolated rabbit cornea. The analysis showed that ruthenium red was found between the cells of the superficial epithelium indicating that 0.001% PQ-1 damaged the corneal tissue (López and Ubels, 1991). In addition, other groups indirectly evaluated the toxicity of 0.001% PQ-1 using *in vitro* cultured human corneal epithelial cells, which were exposed to a mixture that contained 0.001% PQ-1 (Ammar *et al.,* 2010; Paimela *et al.,* 2012). Ammar *et al*. found that travoprost preserved with 0.001% PQ-1 reduced the living cells to 80% after 25 min of incubation (Ammar *et al.,* 2010). A similar investigation also showed that pure 0.001% PQ-1 reduced the percentage of living

cells to about 60% after 30 min of application (Paimela *et al.,* 2012). These studies suggest that PQ-1 can cause a disruption of the superficial rabbit corneal epithelium *in vivo* and has a toxic effect, which leads to the death of about 30% of corneal epithelial cells *in vitro*.

In the current investigation, 0.0005% PQ-1 decreased the TEER values of the HC construct after 30 min of application. Thus, it can be assumed that concentrations higher than 0.0005% PQ-1 have an even stronger effect on the TEER values. In addition, measuring the TEER values also demonstrates that PQ-1 concentrations lower than 0.001% (e.g. 0.0005%) can affect *in vitro* the integrity the barrier function of the HC construct. Furthermore, it can be also assumed that this alteration is due to the toxic effect of PQ-1 as previously proven in cytotoxicity studies *in vitro*.

4.4.1.6 Purite®

The preservative Purite® is a stabilized oxychloro complex with antibacterial, antifungal and antiviral activity (Kaur *et al.,* 2009). Its effect on the HC construct was evaluated for concentrations ranging from 0.00005% to 0.005% (see Figure 4-29), the latter being the concentration used in ophthalmic solutions (Galanopoulos and Goldberg, 2009).

As it was already observed within the scope of the evaluation of other preservatives, all tested concentrations of Purite® (0.00005%, 0.0005%, 0.001% and 0.005%) caused a 25% alteration of the TEER values of the HC construct, which was also noted for preservative-free KRB during the first 10 min of incubation. Interestingly, the TEER values increased slightly until 90 min of the experiments. After this time, the TEER values did not change significantly until the end of the tests. In addition, it can also be seen in Figure 4-29 that there is a relatively high variation in the average TEER values. This can be due to the different expression levels of zona occludens during the cultivation of the HC construct, which has influence on the corneal barrier function of the model.

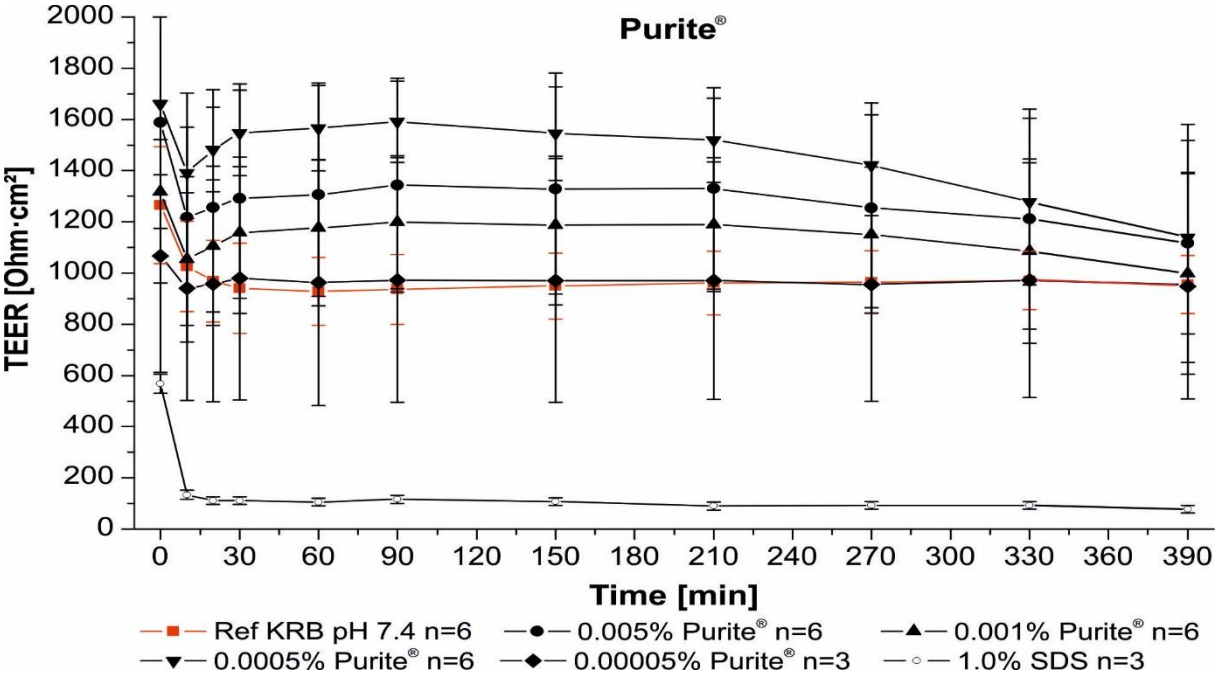

Figure 4-29: Mean of TEER values ± SD of the HC construct in function of time obtained after incubation with several concentrations of Purite® compared to preservative-free KRB

The influence of pure Purite® on TEER has not yet been investigated neither on native corneal tissue nor on cultured corneal cells. The present study is the first to evaluate this effect on the HC construct in *in vitro* experiments. Thus, the current data cannot be directly compared to the aforementioned corneal models. However, one group studied the influence of this preservative indirectly *in vivo* with artificial tears containing 0.005% Purite® (Way *et al.,* 2001). They used scanning electron microscopy to analyze the appearance of the rabbit corneal epithelium after the instillation of the artificial tears with 0.005% Purite® into the eyes of the test animals four times a day for one week. The results showed that 0.005% Purite® did not cause corneal epithelial damage and did not affect the epithelial tight junctions. In contrast, another *in vivo* experiment with rabbits revealed that the long-term administration of the eye drops brimonidine that are formulated with 0.005% Purite® (administered between once and twice on a daily basis for one month) into the eye can have a disruptive effect on the corneal epithelium (Noecker *et al.,* 2004). The evidence indicates that the influence of Purite® on the corneal epithelium can potentially be associated with the duration of the treatment.

In summary, the present work shows that Purite® (in concentrations ranging from 0.00005% to 0.005%) does not affect the barrier function of the HC construct during 390 min of application. Nevertheless, it cannot be ruled out that an exposure time longer than 390 min could lead to a dysfunction of tight junctions of the HC construct.

4.4.1.7 sofZia®

An alternative preservative system, which has recently been introduced into the market, is the buffered preservative system called sofZia®. This buffer system has antibacterial and antifungal activities. The original composition developed by Chowhan *et al.,* 2010, was adapted for the current investigation since HP-Guar is not part of sofZia® preservative system and propylene glycol leads to precipitation of the solution (see section 3.1.5).

The results of this study are shown in Figure 4-30. It can be noted that the TEER values increased after 10 min of application. Interestingly, these values were stable almost until 90 min, thereafter the TEER values decreased with a constant rate of approximately 300 Ohm·cm² per hour until reaching a level of about 240 Ohm·cm² until 390 min of incubation.

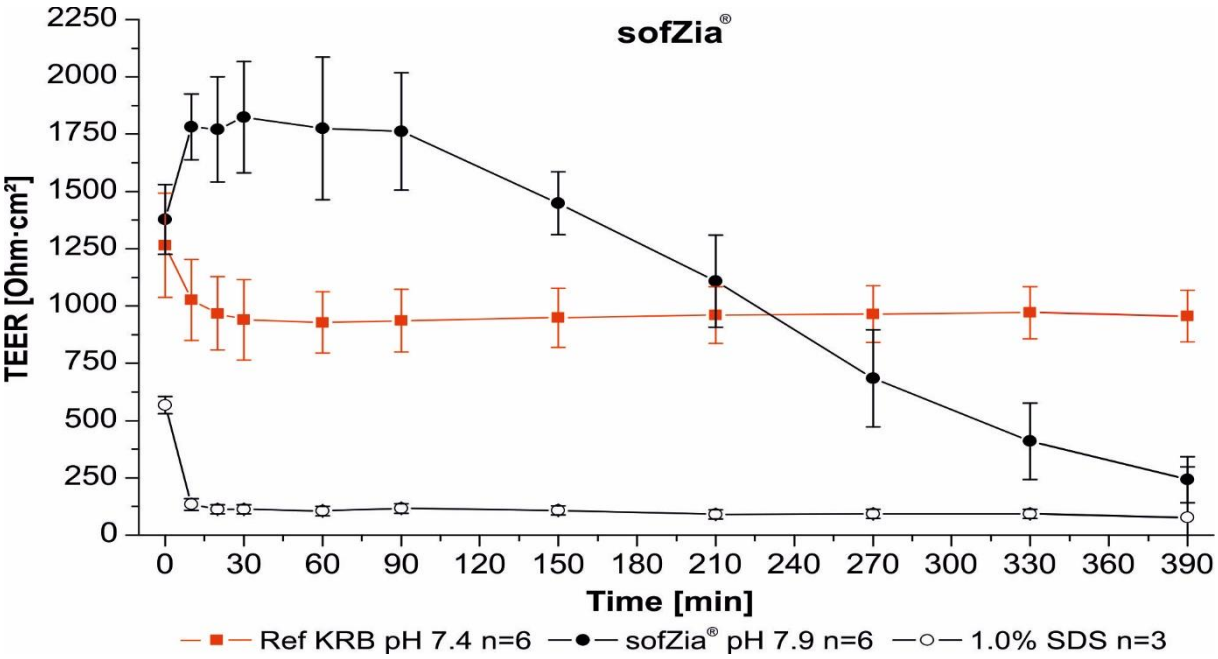

Figure 4-30: Mean ± SD of TEER values of the HC construct in function of time obtained after incubation with sofZia® at pH 7.9 compared to preservative-free KRB at pH 7.4

sofZia® has been recognized by the United States Pharmacopoeia as an alternative preservative system to be used in topical multidose medications and is also considered to be relatively harmless to the corneal surface in the way it is currently used, for instance, in the ophthalmic medication sofZia®-preserved travoprost (Ryan *et al.,* 2011, Noecker, 2007). Nevertheless, the effect of pure sofZia® on the barrier function of the human or rabbit cornea has been poorly investigated until today. As a consequence, no precise TEER values that would indicate how the barrier function is affected by sofZia® have been reported to date. However, the effect of sofZia®-preserved travoprost has been evaluated *in vivo* with regard to the morphology of rabbit cornea. In 2006, Whitson *et al*. demonstrated that neither the application of one drop of travoprost with sofZia® over 3 min nor the application of 1 drop per

min for 3 min resulted in changes of the superficial corneal epithelium or the cell size of the cornea of the test rabbits (Whitson *et al.,* 2006). In addition, in 2008, Kahook and Noecker evaluated the histopathological effect of sofZia®-preserved travoprost with the aforementioned corneal model in a study, which involved the administration of one dose per day for 30 days. In this *in vivo* experiment, they reported no significant damage to superficial cells and concluded that travoprost formulated with sofZia® had only a minimal toxic effect on the corneal surface (Kahook and Noecker*,* 2008). In contrast, *in vivo* studies with human participants involving the long-term application of travoprost with sofZia® for three months revealed that this ophthalmic medication caused the death of small groups of corneal epithelial cells. This effect on the corneal surface was directly attributed to the preservative system sofZia® and not to the active substance travoprost (Aihara *et al.,* 2013). In addition, *in vitro* cytotoxic studies with sofZia® disclosed that this preservatives decreased the percentage of living cells of cultured human corneal epithelial cells to 30% after 25 min of incubation (Kahook *et al.,* 2008). These reports suggest that sofZia® affects the integrity of the corneal barrier to a certain degree.

The data achieved within the scope of current investigation demonstrate that sofZia® does not produce any changes in the HC construct during the first 90 min of application. This suggests that the tight junctions between the superficial cells of the HC construct have not been affected during this time that is similar to the observations made by Whitson *et al*. Nevertheless, a steady decrease of the TEER was measured after this time indicating that sofZia® affected the tight junctions of the HC construct. Thus, these findings confirm that sofZia® can induce alterations in the *in vitro* superficial corneal epithelium as also demonstrated in *in vivo* experiments with rabbits by Aihara *et al*. (Aihara *et al.,* 2013). In addition, this alteration seen in the HC construct can be associated to the low toxic effect that sofZia® revealed on cultured human corneal epithelial cells as Kahook *et al*. observed in *in vitro* experiments (Kahook *et al.,* 2008).

In summary, it can be concluded that the results of the current investigation are consistent with those obtained *in vivo.* Moreover, it can also be assumed that the HC construct displays similar barrier properties as native rabbit tissue when sofZia® is applied for a short time. In addition, the reduction of the TEER values of the HC construct after 90 min was probably caused by the toxic effect sofZia® as previously described by Kahook *et al*. (Kahook *et al.,* 2008).

4.4.2. Influence of preservatives in permeability assays

In this section the permeability of the HC construct to the hydrophilic compound Na-FLU and the non-hydrophilic drug bimatoprost was evaluated under the influence of different preservative concentrations (0.0005% BAC; 0.001% MP, 0.00005% Thio, 0.0005% PQ-1, 0.005% Purite® and sofZia®), which caused minor changes in the TEER values. The effect of

the preservatives on the permeability of the HC construct was evaluated considering the P_{app} values of Na-FLU and bimatoprost. Furthermore, the BAC concentration of 0.005% BAC, which is known to have a negative impact on the barrier function of the HC construct, was used as a positive control to demonstrate that a significant reduction in the TEER values of the HC construct results in an increase of corneal permeability to hydrophilic compounds. The donor solutions containing Na-FLU or bimatoprost as well as the different preservatives were prepared in KBR at pH 7.4, except for the preservative sofZia®, which is a buffer solution at pH 7.9. The resulting P_{app} values of Na-FLU and bimatoprost were compared with those obtained without preservatives. An enhancement factor (Ef) was calculated in order to assess the effect of these preservatives. The Ef was defined as the ratio between the P_{app} of the test substances with preservative and the P_{app} of the test substances without preservative. Finally, the results achieved with the HC construct were compared with those available in the literature in order to evaluate the degree of comparability between the barrier properties of the HC construct and that of native corneal tissue following the exposure to preservatives.

4.4.2.1 Permeation of sodium fluorescein through the HC construct in presence and absence of preservatives

The cumulative transport of Na-FLU in absence and presence of preservatives for ophthalmic use is illustrated in Figure 4-31 and Figure 4-32.

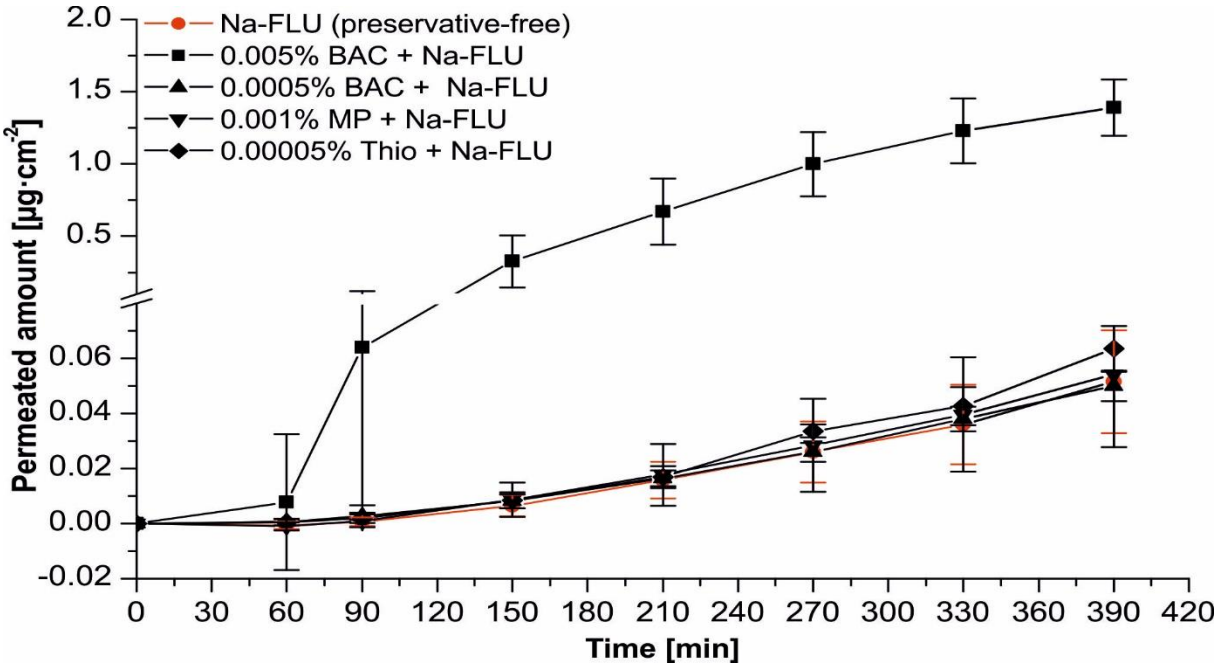

Figure 4-31: Permeated amounts per unit area of sodium fluorescein (Na-FLU) from preservative-free KRB and KRB containing benzalkonium chloride (BAC), methylparaben (MP) and thiomersal (Thio) versus time. Each point shows the mean ± SD of five to six independent experiments.

The permeated amount of Na-FLU under influence of BAC 0.005% was 27-fold higher compared to preservative-free Na-FLU, whereas the permeated amount of Na-FLU in KRB with BAC 0.0005% was almost in the same range as the amount determined for preservative-free Na-FLU. The resulting P_{app} achieved with Na-FLU was significantly increased by 0.005% BAC, which was accompanied by a reduction of the TEER values of the HC construct. In contrast, the P_{app} obtained for Na-FLU was not significantly influenced in the presence of BAC 0.0005% and the TEER values remained constant at about 670 Ohm·cm² (see Table 4-3). Furthermore, MP 0.001% neither affected the permeability of the HC construct to Na-FLU nor the TEER values. Similar results were found for 0.00005% Thio. However, it was noted that the TEER values significantly decreased at the end of the transport experiment (see Figure 4-31 and Figure 4-32).

The P_{app} and TEER values, which were obtained for Na-FLU in combination with various preservatives at different concentrations are summarized in Table 4-3.

Table 4-3: P_{app} and TEER values (mean ± SD) calculated for Na-FLU with and without preservatives

Preservative [%(m/v)]	**Mean ± SD**				**Enhancement Factor**
	TEER [Ohm·cm²]			**P_{app} 10^{-7} [cm·s^{-1}]**	
	Medium	**60 min**	**390 min**		
preservative-free	711 ± 128	674 ± 254	653 ± 194	2.92 ± 0.85	---
0.005% BAC	1075 ± 131	77 ± 8	56 ± 11	76.94 ± 4.66	26.34
0.0005% BAC	1014 ± 130	666 ± 101	679 ± 76	2.76 ± 0.36	0.94
0.001% MP	827 ± 91	786 ± 79	732 ± 56	3.31 ± 1.36	1.13
0.00005% Thio	974 ± 54	612 ± 52	256 ± 19	3.31 ± 0.20	1.13

BAC is well known for its permeation enhancing properties. Moreover, its effect on the corneal permeability, in particular, with regard to hydrophilic substances has frequently been evaluated *in vivo*, *ex vivo* and in *in vitro* experiments with cultured corneal cells (Burstein, 1984; Majundar *et al.,* 2008; Mccarey and Edelhauser, 2007). In earlier *in vivo* studies, the influence of 0.0025%, 0.005%, 0.01%, 0.02% and 0.05% BAC on the permeability of human and rabbit cornea was evaluated based on the permeation of the hydrophilic substance fluorescein (Burstein, 1984). Burstein detected that 0.0025% BAC increased the permeation of fluorescein through rabbit cornea, whereas concentrations higher than 0.0025% BAC led even to a several-fold increase of the permeability of rabbit cornea to fluorescein. In contrast, the permeation of fluorescein through the human cornea was only significantly influenced by concentrations higher than or equal to 0.02% BAC (Burstein, 1984). Another *in vivo* study with rabbits was conducted by Ubels *et al*. They reported that the administration of 0.01% BAC into the eyes of the test animals, where the substance was left for only 5 min, led to an

approximately 13-fold increase of the corneal permeability to the hydrophilic substance 5,6-carboxyfluorescein. Furthermore, the permeation enhancing effect of BAC has already been demonstrated in a series of *ex vivo* experiments with isolated rabbit cornea. In 1995, Sasaki *et al.,* for instance, showed that BAC increased the permeability of isolated rabbit cornea to the hydrophilic paracellular markers FD-4 and FD-10 about 29 and 37-fold, respectively (Sasaki *et al.,* 1995). Another study carried out by Nakamura *et al*. has also demonstrated that BAC 0.002%, 0.01%, 0.05% enhanced the permeability of the rabbit cornea to FD-4 6, 12 and 27-fold, respectively (Nakamura *et al.,* 2007). In fact, the authors have also reported that the increase of the corneal permeability to FD-4 correlated with a decrease of the TEER of the isolated rabbit cornea (Nakamura *et al.,* 2007).

Regarding culture-based corneal models it has previously been reported that BAC concentrations of 0.005% and 0.01% led to a significant increase of the *in vitro* permeability of cultured SIRC epithelial cells to phenylacetyl phenylephone HCl. This alteration in the permeability of the corneal model correlated with a reduction in the TEER (Goskonda *et al.,* 2000).

In short, all these investigations indicate that BAC concentrations ranging from 0.002% to 0.02% exhibit the same concentration-dependent permeation enhancing effect using *in vivo* and *ex vivo* corneal models as well as cultured corneal epithelial cells. Moreover, this effect has been attributed to BAC concentrations, which damage to the corneal barrier or cause a disruption of the tight junctions of the superficial corneal epithelium. In fact, the current investigation also demonstrates that 0.005% BAC leads to a several-fold increase in the corneal permeability of the HC construct to Na-FLU. Moreover, this change in the corneal permeability strongly correlated with a reduction of the barrier function (TEER) of the model. Furthermore, the present work also demonstrated that 0.0005% BAC neither causes a reduction in the barrier function nor changes in the corneal permeability of the HC construct to Na-FLU. Thus, the results of the current study are in line with those previously reported for *in vivo* or *ex vivo* rabbit cornea or culture-based corneal models.

The effect of pure MP on the corneal permeability has not yet been investigated within the scope of *in vivo* or *ex vivo* studies with rabbit cornea. Only a small number of investigations, either *in vivo* studies with rabbits or *in vitro* cultured corneal models (see section 4.4.1.3), have focused on investigating the corneal integrity after the long-term administration of MP (0.005%) or demonstrating the toxic effect of MP. For this reason, the results obtained with the HC construct are not comparable to those achieved with the above-mentioned corneal models. The present study showed that 0.001% MP did not change the corneal permeability of the HC construct to Na-FLU. Thus, it can be suggested that the tight junctions of the HC construct are highly resistant to 0.001% MP.

In vivo experiments with rabbits conducted by Fister and Burstein in 1975 demonstrated that Thio 0.01% led to the desquamation of epithelial cells with ocular lesions (epithelial holes) after 6 h of application. Based on this observation, a significant modification of the corneal permeability at least to hydrophilic substances can be expected as has already been demonstrated by López Bernal and Ubels in 1991 in *in vivo* experiments with rabbits (López Bernal and Ubels, 1991). They have described that 0.001% and 0.004% Thio almost doubled the permeability of the corneal epithelium of rabbits to 5,6-carboxyfluorescein after 180 min. Following the permeation experiments, the rabbit cornea was isolated and stained with ruthenium red. Based on the morphological evaluation of the stained corneal tissue, the group was able to correlate the increase of corneal permeability with a damage of the superficial corneal epithelium since they found ruthenium red in the first layers of the superficial corneal epithelium (López Bernal and Ubels, 1991). Thus, Thio at concentrations higher than or equal to 0.001% can result in an alteration of the superficial corneal epithelium of rabbits.

The current study also indicates that the barrier function of the HC construct is equivalent to native rabbit corneal tissue. This conclusion is based on the observation that similar Thio concentrations significantly affected the barrier function of the HC construct. Moreover, it was found that lower concentrations of Thio, e.g. 0.00005%, did not increase the corneal permeability of the HC construct to Na-FLU. This can be explained by the fact that 0.00005% Thio did not damage the barrier function of the HC construct significantly since the TEER values were not substantially altered.

The individual effect of the preservatives PQ-1, Purite® and sofZia® on the permeation of Na-FLU through the HC construct is represented in Figure 4-32. The results demonstrate that 0.0005% PQ-1 increased the transported amount of Na-FLU 1.9-fold, whereas the transported amounts of Na-FLU in combination with 0.005% Purite® and sofZia® were similar to that of Na-FLU in preservative-free KRB. PQ-1 in concentration of 0.0005% led to alterations in the TEER values of the HC construct that was expressed in a reduction of approx. 40% after the preincubation time (60 min) and then a further reduction of 20% at the end of the permeation study (see Table 4-4). This alteration in the TEER values correlated well with an increase of the P_{app} of Na-FLU, which was significant higher in comparison with the P_{app} of Na-FLU without preservatives. No modification of the TEER values was observed for the preservative Purite® with Na-FLU in KRB. For the preservative sofZia® it can be pointed out that the TEER values of the HC construct were increased 15% after the preincubation time and gradually reduced to 50% until the end of the permeability study. However, TEER reduction didn't result in any alteration of the transport of Na-FLU through

the HC construct. The P_{app} and TEER values, which were obtained for Na-FLU in combination with 0.0005% PQ-1, 0.005% Purite® and sofZia® are summarized in Table 4-4.

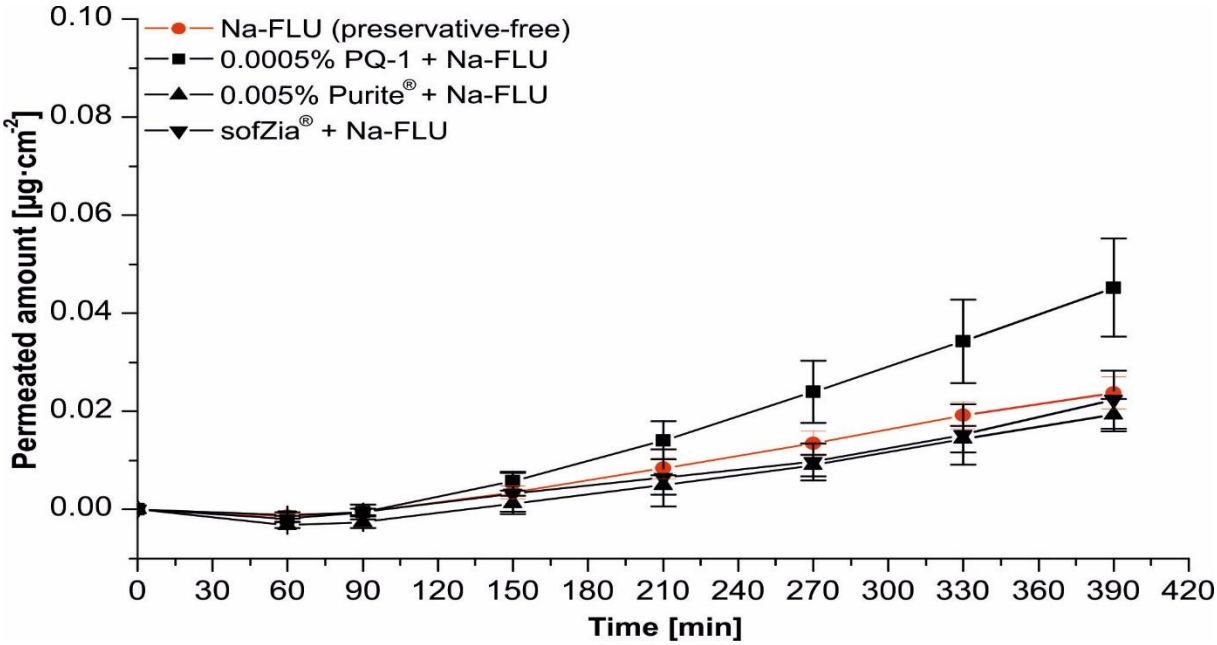

Figure 4-32: Permeated amounts per unit area of sodium fluorescein (Na-FLU) from preservative-free KRB and KRB containing Polyquad® (PQ-1), Purite® and sofZia® versus time. Each point represents the mean ± SD of five to six independent experiments.

Table 4-4: P_{app} and TEER values (mean ± SD) calculated for Na-FLU with and without preservatives

Preservative [%(m/v)]	**Mean ± SD**				**Enhancement Factor**
	TEER [Ohm·cm²]			**P_{app} 10^{-7} [cm·s^{-1}]**	
	Medium	**60 min**	**390 min**		
preservative-free	1303 ± 335	861 ± 184	1016 ± 159	1.80 ± 0.30	---
0.0005% PQ-1	1480 ± 99	846 ± 141	524 ± 108	3.13 ± 0.78	1.83
0.005% Purite®	1027 ± 216	853 ± 203	952 ± 253	1.50 ± 0.26	0.83
sofZia®	1372 ± 129	1577 ± 238	692 ± 215	1.44 ± 0.25	0.80

Even though PQ-1, Purite® and sofZia have been developed as potential alternatives to the older generation of preservatives, such as BAC, Ce or MP, their effect on the corneal permeability to ophthalmic drugs has been poorly or not at all investigated up to now. The current study is the first *in vitro* study to investigate the influence of the above-mentioned preservatives on the corneal permeability of the HC construct to the hydrophilic substance Na-FLU. The permeation of Na-FLU was evaluated on the basis of changes in the TEER that were measured in real time within the scope of transport experiments. However, since it is the first investigation, the TEER and permeability data cannot be directly compared with

those obtained in *in vivo* or *ex vivo* experiments with rabbit cornea or with cell culture-based corneal models.

Regarding PQ-1, López Bernal and Ubels investigated the influence of 0.001% PQ-1 on the corneal permeability of rabbit eyes *in vivo* (López Bernal and Ubels*,* 1991). They found that 0.001% PQ-1 increased the permeability of the rabbit cornea to 5,6-carboxyfluorescein a compound with similar physicochemical characteristics as Na-FLU, about 3-fold after 90 min and about 6-fold after 180 min in comparison to the permeability of untreated rabbit cornea. Likewise, this group found that the alteration in the corneal permeability was caused by the disruption of tight junctions.

In the current study, it has been demonstrated that 0.0005% PQ-1 influences the corneal permeability of the HC construct to Na-FLU by a factor of approximately 1.8 after 390 min. This change in the corneal permeability has been associated with a dysfunction of the tight junctions between the superficial epithelial cells. The findings allow the assumption that 0.001% PQ-1 affects the corneal permeability of the HC construct to Na-FLU to an extent, which is comparable to that observed by López Bernal and Ubels for rabbit corneal tissue.

Unfortunately, to my knowledge, no data have yet been reported for Purite® regarding its influence on the permeability of native corneal tissue. In the present study, it has been noted that Purite® does not affect the corneal permeability of the HC construct to Na-FLU in comparison to that of the untreated HC construct.

With regard to sofZia®, in 2007, Mccarey and Edelhauser investigated the effect of sofZia®-preserved travoprost on the permeability of rabbit cornea in *in vivo* experiments. The data were compared with those collected on untreated animals in order to determine changes in the corneal permeability to 5,6-carboxyfluorescein (Mccarey and Edelhauser*,* 2007). They found that the rabbit cornea when being exposed to sofZia®-preserved travoprost for 3 min did not change its permeability to 5,6-carboxyfluorescein compared to that of the cornea of untreated animals. After the permeation experiments the rabbit cornea was isolated, stained with ruthenium red and analyzed under the transmission electron microscope. They noted that the cornea treated with sofZia®-preserved travoprost showed no significant alteration with regard to the tight junctions. This investigation indicates that the permeability of the rabbit cornea is not significantly affected when the cornea is exposed to sofZia® for only a short time.

In the current investigation, it was observed that the TEER of the HC construct decreased steadily after 90 min of exposure to sofZia® with Na-FLU. Nevertheless, the permeability of the HC construct to Na-FLU was comparable to its permeability before the application of sofZia®. Thus, the current results are in good agreement with those previously reported for

native rabbit cornea. In addition, the data also indicate that sofZia® produced a dysfunction of the tight junctions of the HC construct, which was dependent on the time of exposure. However, the alteration seen in the barrier function did not exceed the limit TEER value of 400 Ohm·cm² neither after 60 min nor 390 min.

4.4.2.2 Permeation of bimatoprost through the HC construct in presence and absence of preservatives

Bimatoprost was selected as a model drug to investigate the influence of the tolerated preservative concentrations (BAC 0.0005%, MP 0.001%, Thio 0.00005%, PQ-1 0.0005%, Purite® 0.005% and sofZia®) on the permeability of the HC construct. The effect caused by these preservative concentrations was evaluated by comparing the P_{app} values of bimatoprost in preservative-free and preservative containing KRB.

The cumulative transport of bimatoprost from KRB containing different preservative concentrations is represented in Figure 4-33 and Figure 4-34. The illustrations show that the transported amounts of bimatoprost did not differ significantly between the experiments carried out in the absence or in the presence of preservatives. Moreover, the comparison of the P_{app} values of bimatoprost expressed as enhancement factor indicated that none of these preservative concentrations were capable to alter the permeability of the HC construct to bimatoprost (see Table 4-5 right column).

Donor solutions containing bimatoprost and 0.00005% Thio, 0.0.005% PQ-1 or sofZia®, respectively, altered the TEER values to some extent. However, none of these alterations affected the permeability of HC construct to bimatoprost. This can be explained by the fact that the passive transcellular drug diffusion through the HC construct is fewer dependent on the TEER values than observed for hydrophilic compounds, as was demonstrated during the prevalidation phase of the HC model (Hahne *et al.,* 2012).

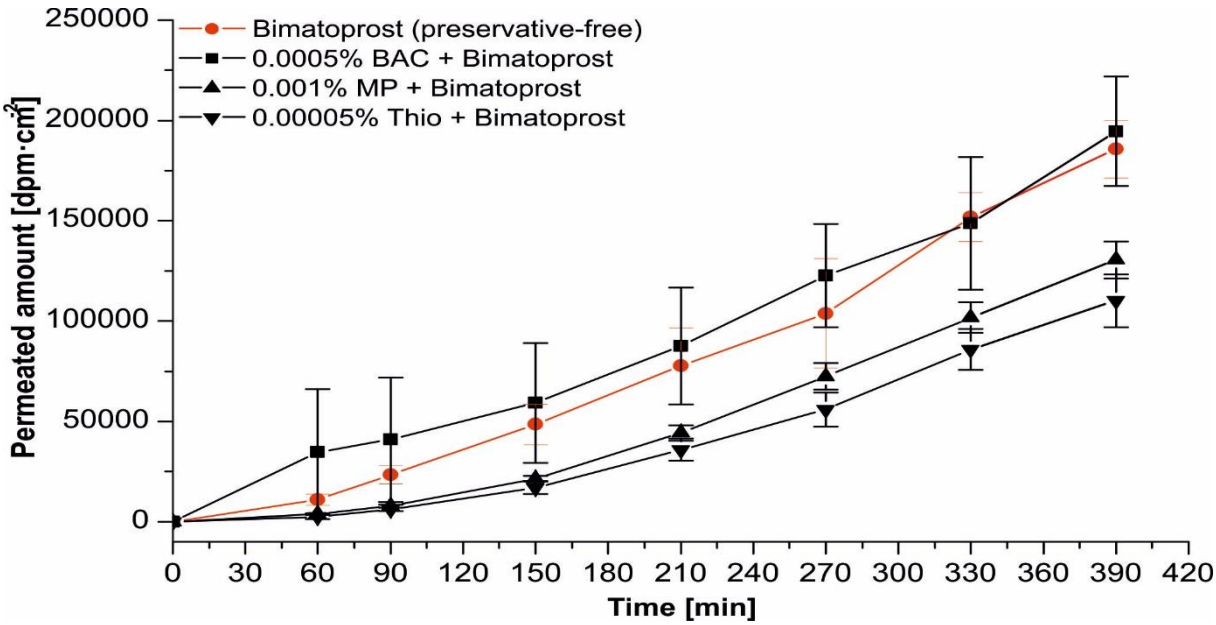

Figure 4-33: Permeated amounts per unit area of bimatoprost from preservative-free KRB and KRB containing benzalkonium chloride (BAC), methylparaben (MP) and thiomersal (Thio) versus time. Each point shows the mean ± SD of five to six independent experiments.

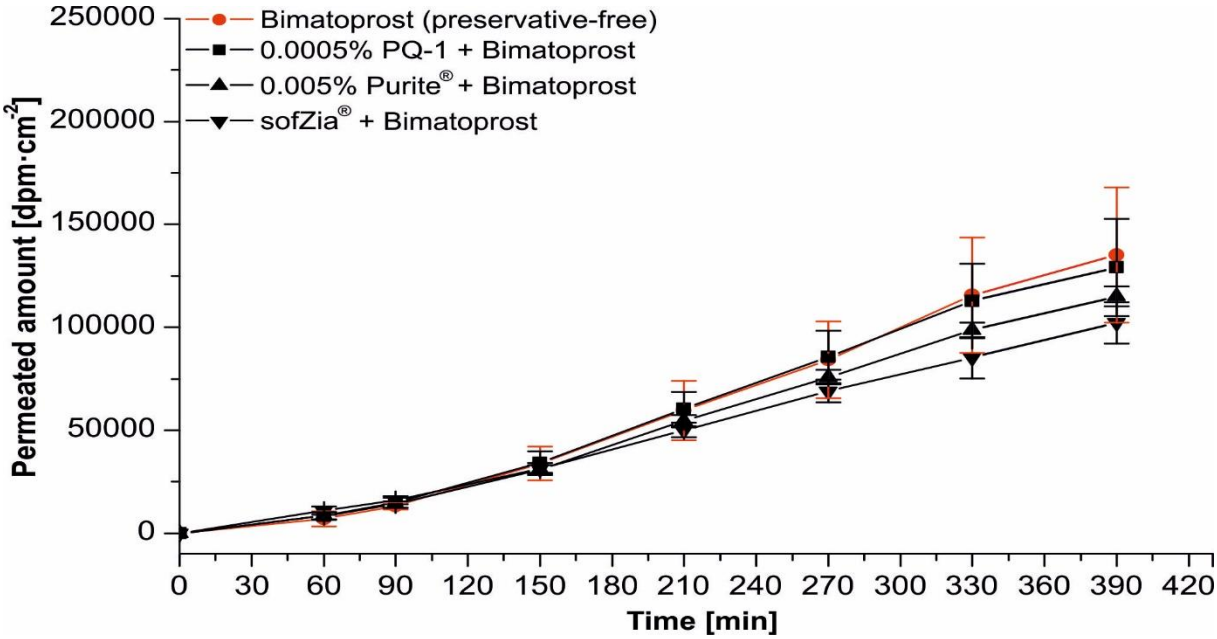

Figure 4-34: Permeated amounts per unit area of bimatoprost from preservative-free KRB and KRB containing Polyquad® (PQ-1), Purite® and sofZia® versus time. Each point shows the mean ± SD of five to six independent experiments.

Table 4-5: P_{app} and TEER values (mean ± SD) calculated for bimatoprost and enhancement factors of various concentrations of different preservatives

Preservative [%(m/v)]	Mean ± SD				Enhancement Factor
	TEER [Ohm·cm²]			P_{app} 10^{-7} [cm·s^{-1}]	
	Medium	60 min	390 min		
preservative-free	1102 ± 288	838 ± 100	1061 ± 245	39.45 ± 10.42	---
0.0005% BAC	860 ± 63	706 ± 96	735 ± 31	39.35 ± 3.09	1.00
0.001% MP	885 ± 156	610 ± 93	689 ± 107	37.75 ± 1.56	0.96
0.00005% Thio	883 ± 65	610 ± 56	280 ± 32	39.77 ± 3.59	1.00
preservative-free	917 ± 95	846 ± 117	856 ± 106	24.16 ± 1.59	---
0.0005% PQ-1	1214 ± 155	626 ± 78	330 ± 62	24.62 ± 1.04	1.01
0.005% Purite®	1101 ± 118	946 ± 118	1032 ± 113	24.87 ± 0.70	1.02
sofZia®	1023 ± 178	1264 ± 311	592 ± 223	23.34 ± 2.94	0.97

Prostaglandins analogs such as travoprost, latanoprost, tafluprost (prostanoids) and bimatoprost (prostamide) are used for the treatment of glaucoma. The majority of the commercially available ophthalmic solutions contain preservatives. As already mentioned above, preservatives like BAC additionally work as permeation enhancers, which can increase the concentration of an ophthalmic drug in the aqueous humor, in particular of hydrophilic substances (Majumdar *et al.,* 2008; Mccarey and Edelhauser*,* 2007). The effect of BAC on the transcorneal permeation of lipophilic drugs, such as bimatoprost, timolol maleate and dexamethasone, has already been investigated. Chang *et al*. demonstrated in *in vivo* and *in vitro* experiments using rabbit cornea and primary cultures of rabbit corneal epithelial cells (pRCE) that increasing of BAC concentration from 0.005% to 0.02% significantly influenced the permeation rate of bimatoprost (0.03%) through *in vivo* as well as *ex vivo* corneal tissue (Chang *et al.,* 2012). Furthermore, Saettone and co-workers reported that 0.02% BAC increased the permeation rate of timolol maleate through excised rabbit cornea 2.6-fold (Saettone *et al.,* 1996). Another group described that 0.01% BAC increased the permeability of *ex vivo* rabbit cornea to the lipophilic drug dexamethasone 3-fold (Camber and Edman*,* 1987). All these reports indicate that BAC concentrations of 0.01% and higher can change the permeability of rabbit cornea to lipophilic drugs.

The current study has demonstrated that 0.0005% BAC neither causes a significant change in the TEER values nor increases the permeability of the HC construct to bimatoprost. Furthermore, it has also been shown that BAC concentrations higher than 0.0005% (e.g. 0.005%) can affect the barrier function of the HC construct. Therefore, based on the collected evidence, it can be expected that BAC concentrations higher than or equal to 0.005% (e.g. 0.01%) also affect the permeability of the HC construct to bimatoprost.

Besides the current study with the HC construct, no other investigation has yet been conducted to evaluate the effect of pure MP, Thio, PQ-1, Purite® and sofZia® on the corneal permeability to bimatoprost. Hence, the data obtained with the HC construct for bimatoprost in the presence of the preservatives cannot directly be compared with results achieved with *in vivo*, *ex vivo* rabbit cornea or cultured corneal cells.

4.4.3. Summary of preservative effects on the HC construct

The majority of the commercially available ophthalmic solutions are formulated with preservatives to prevent microbial contamination. In the light of the fact that some preservatives have been reported to cause epithelial damage (Ahmad and Chishti, 1992; Epstein *et al.,* 2009; Ayaki and Iwasawa, 2010) and increase the ocular bioavailability of drugs by influencing the corneal permeability, the first aim of this chapter was to investigate the effect of preservatives on the epithelial integrity of the HC construct by measuring the transepithelial electrical resistance. The second aim of this chapter was to evaluate the effect of preservatives on the permeability of the HC construct expressed as permeation coefficients of sodium fluorescein as a hydrophilic marker substance and bimatoprost (non-hydrophilic) as a model drug. The data obtained with the HC construct were compared with those from *in vivo, ex vivo* and *in vitro* experiments available in the literature.

The present *in vitro* study has demonstrated that the effect of preservatives (BAC, Ce, Thio, MP, PQ-1, Purite® and sofZia®) on the barrier function of the HC construct basically depends on the concentration and the exposure time. Furthermore, it was found that in particular the effect of BAC, Thio, MP and PQ-1 on the epithelial integrity of the HC construct is intensified by increasing the concentration used. For instance, BAC concentrations higher than or equal to 0.005% resulted in TEER value reduction after the first 10 min of application. In contrast, 0.001% BAC required 90 min to cause a substantial reduction of TEER. Moreover, BAC concentrations lower than 0.001% (e.g. 0.0005%) had no effect on the barrier properties of the HC construct. In previously published studies it has been found that similar or identical concentrations of BAC and Thio led to a concentration and time-dependent reduction of TEER values of *ex vivo* rabbit cornea (Chetoni *et al.,* 2003; Nakamura *et al.,* 2007; Burstein and Klyce, 1977). Furthermore, the data obtained in this study are in line with results of previous *in vivo* investigations that evaluated the effect of PQ-1 and MP on rabbit corneal epithelium. It was observed in the present study that PQ-1 concentrations lower than 0.0005% had no effect on TEER of the HC construct. In contrast, 0.0005% PQ-1 affected the TEER of the HC construct even after 30 min of application. This implies that 0.001% PQ-1, the commonly used concentration in ophthalmic drugs, can accelerate the TEER reduction of the HC construct. This assumption is supported by the data reported by Mohamed *et al.,* who found in *in vivo* experiments that the application of ophthalmic solutions containing 0.001%

PQ-1 into rabbit eyes caused TEER reduction of about 20% after 1 min of exposure (Mohamed *et al.,* 2006). Moreover, it was observed in the present study that 0.1% MP decreased the TEER rapidly after the first 10 min of incubation, whereas only a slight but steady TEER value reduction was noted for 0.005% MP after 30 min of application. Furthermore, 0.001% MP did not change the barrier properties of the HC construct. Unfortunately, it was not possible to directly compare the data obtained with the HC construct with those of animal cornea since the effect of MP on the TEER of rabbit cornea has not yet been reported. Nevertheless, a suitable and frequently used approach to demonstrate the effect of substances on the corneal surface is the morphological examination by means of microscopic analysis. For example, Becquet *et al.* used this approach to demonstrate that 0.005% MP produces morphological alterations in the corneal epithelium of rats (Becquet *et al.,* 1997).

With regard to the other preservatives (Ce, Purite® and sofZia®), the effect on the TEER values of native corneal tissue has not been investigated so far. In contrast, the effect of these preservatives on the structure of superficial epithelium of rabbit cornea has already been evaluated by other groups in *in vivo* experiments (Way *et al.,* 2001; Mahmoud*,* 2007; Mahmoud *et al.,* 2011; Whitson *et al.,* 2006; Kahook and Noecker*,* 2008). These morphological studies have proven that Ce and Purite® concentrations lower than or equal to 0.02% (Ce) and 0.005% (Purite®) as well as the short-term application of sofZia®-preserved ophthalmic solutions are relatively harmless to rabbit corneal epithelium; a conclusion, which corresponds to the results obtained with the HC construct.

Furthermore, it can be suggested that the alterations of the barrier function of the HC construct caused by the preservatives were due to their cytotoxic potential. This assumption is supported by the results of several cytotoxicity studies reported by other groups (Kusano *et al.,* 2010; Nakamura *et al.,* 2007; Ayaki *et al.,* 2007; Matsuda *et al.,* 2009, Saarinen-Savolainen *et al.,* 1998; Epstein *et al.,* 2009; Tripathi *et al.,* 1992; Ammar *et al.,* 2010; Ammar *et al.,* 2011; Paimela *et al.,* 2012; Zheng *et al.,* 2015). These investigations have demonstrated that the reduction of the percentage of living corneal epithelial cells can be attributed to the cytotoxic properties of preservatives, the tested concentrations as well as the exposure time.

Regarding the effect of preservatives on the transcorneal permeation of substances, the present study proved that only preservative concentrations, which affected the tightness of the HC model within the first 30 min of exposure (e.g. BAC (0.005%) and PQ-1 (0.0005%)) also caused a significant increase of the permeability of the HC construct to hydrophilic substances (Na-FLU). This effect has already been observed for rabbit cornea in *in vivo* and *ex vivo* experiments, when corneal tissue was exposed to concentrations higher than or equal to 0.001% BAC or concentrations higher than 0.0005% PQ-1 (Sasaki *et al.,* 1995;

Nakamura *et al.,* 2007; López Bernal and Ubels*,* 1991; Burstein*,* 1984). In contrast, 0.0005% PQ-1 or diluted BAC concentrations (e.g. 0.0005%) did not alter the permeability of the HC construct to bimatoprost. Permeability experiments with lipophilic substances in the presence of preservative concentrations, which significantly affected the tightness of the HC construct were not carried out. Nevertheless, it has been previously demonstrated that 0.002% BAC changed the permeability of rabbit cornea *in vivo* and pRCE cells *in vitro* to bimatoprost (Chang *et al.,* 2012). Other groups also detected that BAC concentrations ranging from 0.01% to 0.025% increase the permeability of *ex vivo* rabbit cornea to lipophilic substances, such as dexamethasone and timolol maleate (Camber and Edman*,* 1987; Ashton *et al.,* 1991).

In short, all evidence reported in the relevant literature and results obtained within the scope of this study suggest that the HC construct behaves in a manner, which is comparable to that already reported for *in vivo* and *ex vivo* rabbit cornea with regard to the effect of preservatives on the permeability properties of the corneal epithelium.

5. Final discussion

5.1.1. Transferability of methods, quality and prevalidation of the HC construct

During the development of new or reformulated ophthalmic drug delivery systems, *in vitro* corneal cell culture models are used as an alternative to excised animal or human cornea within the scope of drug permeability studies. The human Hemicornea model (Hemicornea, HC or Hemicornea construct) has been developed by Reichl and co-workers for the purpose of performing such studies (Hahne and Reichl*,* 2011). This HC construct was the subject of the current investigation.

The HC construct has to fulfill certain criteria before it can be accepted by authorities. The prevalidation of the HC construct is one of these criteria. The prevalidation step involves three main phases: i) protocol refinement, ii) protocol transfer and, iii) protocol performance (Curren *et al.,* 1995). The first phase, including the development of the protocols for constructing the HC construct and methods for performing drug permeability studies, was already carried out by Hahne and Reichl (Hahne and Reichl*,* 2011). Thus, the current chapter deals with the evaluation of the protocol transfer and the performance for the prevalidation of the HC construct carried out at ACB.

In the current study, it was possible to produce several batches of the HC construct with a similar range of TEER, which were stable over an extended period of time (cultivation protocol) and a similar range of permeability to the hydrophilic substance Na-FLU that demonstrates the reproducibility of the barrier function of the HC construct. In addition, acceptance criteria were defined at ACB based on the barrier properties of the HC construct. Thus, in order to ensure that the HC construct possesses sufficient strong barrier properties to be suitable for drug permeability studies, the TEER should be ≥ 400 Ohm·cm² and the P_{app} achieved with Na-FLU should be < $1 \cdot 10^{-6}$ cm·s^{-1}. However, there are currently no generally applicable criteria, which could be used as a basis to assess the suitability of an *in vitro* human corneal model for such studies. This is the first study of this kind, which has been conducted to define such criteria. Nevertheless, these criteria can be compared with those previously reported for others cultivated epithelial cells, such as bronchial and or intestinal cells (Caco-2). The latter is an alternative *in vitro* model, which has already been accepted by validation authorities as a substitute for human intestinal permeability studies (Larregieu and Benet*,* 2013.) Thus, it has been reported that a minimum TEER value of approximately 250 Ohm·cm² confirms the differentiation state of the epithelial barrier and should be adequate to achieve P_{app} values in the order of 10^{-7} cm·s^{-1} for the hydrophilic permeation marker sodium fluorescein (Ehrhardt *et al.,* 2002; Verstraelen and Reichl*,* 2013).

In the current study, the TEER and P_{app} values (Na-FLU) obtained by all participating laboratories (TUBS, UKE and ACB) were plotted to specify a general acceptance criteria for the HC construct for its use in drug permeability studies. The data suggest that a minimum TEER value of 440 Ohm·cm² is required to confirm the barrier properties of the reconstructed corneal epithelium (Hahne *et al.,* 2012). Thus, the current study indicates that the acceptance criteria are of vital importance in order to ensure the high quality of the HC construct at all times. In addition, they are critical parameters to ensure that the HC construct complies with the quality standards of drug permeability studies.

Drug permeability studies are frequently performed using isolated animal cornea, in particular of rabbit origin. However, in general the variability of the results obtained from animals is high (Becker *et al.,* 2008; Anand and Mitra, 2002). Therefore, another objective of this investigation was to demonstrate that the reproducibility of data can be improved by standardizing the methods for cultivating the HC construct and the protocols to perform drug permeability studies. The analysis of the variances of P_{app} values obtained with different substances and HC batches cultivated at the three laboratories reveals that the intralaboratory reproducibility of the HC construct is higher than that of isolated rabbit cornea. However, even though the interlaboratory reproducibility is acceptable, it could be further improved. Until today, there are neither regulatory guidelines nor acceptance criteria for the validation of *in vitro* alternative methods for corneal permeability studies. However, the data obtained with the HC construct can be compared with the Caco-2 cell model. In accordance with the biopharmaceutical classification system and FDA recommendations, the *in vitro* cultured Caco-2 cells can be employed for the screening of new drugs since the permeability data are comparable to those of *in vivo* or *ex vivo* human or animal (in particular from pig) intestinal tissue (FDA Guidance For Industry*,* 2015; Volpe*,* 2008a). The Caco-2 cell model has high intralaboratory reproducibility but a low interlaboratory reproducibility since the protocols used by the laboratories are not well standardized. Furthermore, uncharacterized samples of Caco-2 cells from different origins are often used (Volpe*,* 2008b; Volpe *et al.,* 2007). In addition, it has been recommended that an *in vitro* model with good intralaboratory reproducibility should have a coefficient of variation not higher than 50% and the ratio of the average P_{app} values has to be in the range of 0.5 to 2.0 in order to ensure good interlaboratory variability (Zucco *et al.,* 2005; Prieto *et al.,* 2010). The standardized culture methods and protocols to conduct drug permeability studies that are presented in the current study are in line with the observations made when using the Caco-2 cell model since it has been demonstrated that there is a correlation between the use of standardized protocols and the good intra-interlaboratory reproducibility of the permeability data obtained with the HC

construct. In addition, the above recommended range of values for other validation studies is also in agreement with the data obtained within the scope of the current study.

According to FDA Guidance For Industry an alternative *in vitro* cell model has to fulfill certain criteria to be accepted as a substitute for animal experiments in order to evaluate the permeation of substances through epithelial membranes. One of these criteria is that the vitro model has to be able to differentiate between low and high permeability rates of substances. Another criterion is the comparability of data between different sets of experiments (FDA Guidance For Industry*,* 2015). The permeation data achieved with the HC construct within the scope of the current investigation demonstrate that the HC is able to discriminate between substances depending on their physicochemical properties. Moreover, the reproducibility of data obtained with different HC batches is high. This suggests that the HC construct also complies with the recommendations included in the FDA Guidance For Industry.

The current work provides specifications and acceptance criteria for the development and implementation of well standardized methods for the cultivation of an *in vitro* HC construct as well as protocols for conducting drug permeability experiments. Furthermore, the successful transfer of culture protocols, the high intralaboratory and the sufficiently high interlaboratory reproducibility demonstrate that the prevalidation of the model has been successful. Against this background, it can be concluded that the current work provides the necessary information to consider the HC construct as an alternative model for regulatory biowaiver applications.

5.1.2. Comparative analysis of transcorneal drug absorption

In the last 20 years, *in vitro* ocular models have emerged as substitutes for *in vivo* or *ex vivo* animal experiments to evaluate the transport of substances through the ocular barriers (Hornof *et al.,* 1995; Reichl *et al.,* 2011). Regarding topically applied drugs, the cornea has been identified as the main barrier substances have to pass in order to enter the aqueous humor (Huang *et al.,* 1989; Järvinen *et al.,* 1995). Thus, before new ophthalmic medications can be administered to humans, their transcorneal permeation needs to be evaluated. The permeability properties of the human cornea are attributed to its different cellular layers (epithelium, stroma, and endothelium). It has been demonstrated that the corneal endothelium does not have much influence on the corneal permeability to hydrophilic or lipophilic substances. In contrast, the hydrophilic stromal tissue impairs the transport of substances with lipophilic characteristics and the multilayered epithelium is the main barrier to hydrophilic substances (Huang *et al.,* 1989; Prausnitz and Noonan*,* 1998). Against the background of these previous findings, an *in vitro* corneal tissue (HC construct) of human origin has been reconstructed (Hahne and Reichl*,* 2011) with an equivalent stromal cell layer,

which contains immortalized human keratocytes (Zorn-Kruppa *et al.,* 2005) and an equivalent multilayered corneal epithelium using an immortalized human corneal epithelial cell line (Araki-Sasaki *et al.,* 1995).

Due to the lack of predefined specifications and acceptance criteria for the evaluation of the suitability of *in vitro* corneal models for drug permeability studies, the recommendations of an FDA Guidance For Industry should be followed, which concerns the investigation of the oral permeability of drugs through the intestinal epithelium using *in vitro* cell models, such as the Caco-2 and MDCK cell model (FDA Guidance For Industry*,* 2015). This guidance outlines the evaluation of the passive transport of 20 substances in order to define the permeability class of substances. Another requirement is that the *in vitro* cell culture model has to be able to differentiate between low and high permeable substances on the basis of their physicochemical characteristics. In addition, the permeability data obtained with the *in vitro* models should correlate with the permeability values achieved for human tissue.

Within the scope of the current study, the suitability of the HC construct as a substitute for animal experiments was investigated. For this purpose, the HC construct was used to evaluate the passive permeation of eighteen substances. It was seen that the permeability of the HC construct increased in a sigmoidal manner when the lipophilicity of substances increases. However, an influence of the molecular weight of substances in the range from 60 to 480 $g \cdot mol^{-1}$ on permeation rate was not detected. These observations indicate that the permeability of the HC construct is influenced by the physicochemical properties of substances to the same extent as excised rabbit cornea (Wang *et al.,* 1991; Suhonen *et al.,* 1991, 1996; Hämäläinen *et al.,* 1997; Prausnitz and Noonan*,* 1998). Furthermore, the permeability data obtained for the eighteen substances prove that the HC construct is able to differentiate the permeation of substances based on their physicochemical properties, which is a criterion included in the above-mentioned FDA Guidance For Industry. Moreover, the current HC construct offers several advantages in comparison with previously developed *in vitro* corneal models for drug permeability studies, e.g. its components include an *in vitro* multilayered corneal epithelium and a stromal equivalent, which have barrier properties similar to those of the human cornea (Hahne and Reichl*,* 2011). Due to these components, the HC construct is able to distinguish between low and high permeable substances. Another advantage is that the HC construct can be easily cultured and the reproducibility of its barrier properties between the individual batches is remarkably high. In addition, the tightness of the reconstructed corneal epithelium is in the range of that reported for *in vivo* rabbit cornea (Uematsu *et al.,* 2007; Kusano *et al.,* 2010) as well as for *ex vivo* rabbit and human cornea (Nakamura *et al.,* 2007; Kikuchi *et al.,* 2005; Becker *et al.,* 2008). In contrast, other *in vitro* corneal models only consist of a reconstructed epithelium that is based on, for example, SIRC or CEPI cell lines, which exhibit low permeation barriers to highly hydrophilic and

lipophilic substances, such as mannitol and testosterone, in comparison to excised bovine cornea (Reichl*,* 2008). Similar results have also been reported for the reconstituted human corneal epithelium from Skinethic laboratories and the Epiocular model from MatTek Corporation (Reichl*,* 2008). Moreover, the permeation data obtained with these corneal models indicate that they are not suitable for drug permeability studies since their epithelial tightness is out of the range reported for native corneal tissues (Kikuchi *et al.,* 2005; Nakamura *et al.,* 2007; Uematsu *et al.,* 2007; Reichl*,* 2008, Becker *et al.,* 2008; Kusano *et al.,* 2010). Besides the HC construct used for the present investigation, the Clonetics human corneal epithelium (cHCE) from Cambrex is another *in vitro* corneal model, which is probably suitable for drug permeability studies. The cHCE model possesses a tight epithelial barrier and has similar permeability properties with regard to hydrophilic and lipophilic substances as those reported for isolated native tissue (Becker *et al.,* 2008; Xiang *et al.,* 2009). Despite of the above described advantages of the HC construct in comparison to other models, the main disadvantage of the HC construct is that the TEER values only remain stable for a short period of time of about 3 days (from day 10 to day 13). After this time, the TEER level decreased rapidly. To test the hypothesis that the superficial cells do not receive enough nutrients, 50 µL of KGM were applied to the apical surface of the HC construct. Nevertheless, the TEER continued to decrease constantly after the aforementioned time period. Therefore, the factors, which lead to a decrease of the TEER of the HC construct are not clear. Further studies need to be conducted in order to improve the long-term stability of the barrier properties of the HC construct.

In addition, the passive diffusion of substances with different physicochemical properties obtained with the HC construct has been correlated with relevant data previously reported for *ex vivo* rabbit and human cornea in order to evaluate the equivalence between the permeability of the HC construct and native tissue. The correlation coefficient obtained from the linear regression indicates that the permeability properties of the HC construct and *ex vivo* rabbit cornea are in the same range. However, the slope factor (1.29) indicates that the permeability of *ex vivo* rabbit cornea is generally higher than that of the HC construct. Furthermore, the results show that the permeation of hydrophilic substances, such as Na-FLU, mannitol or acyclovir is nearly identical to that reported for *ex vivo* rabbit tissue, indicating that the permeability characteristics of the corneal epithelium are similar. However, the higher the lipophilicity of substances is (e.g. dexamethasone, metoprolol, rhodamine B), the larger are the differences between the models. This can be explained by the morphological differences between them, in particular thickness and structure of the hydrophilic stroma. Other reasons may also be that the tissue quality and the methodologies used to evaluate the permeation of substances differed between laboratories. Furthermore, it

has to be mentioned that different expression levels of drug-metabolizing enzymes (e.g. cytochrome P450 (Kölln and Reichl, 2016a, 2016b)) and transport proteins, such as P-glycoprotein (P-gp or MDR1), lung resistance-related protein (LRP), breast cancer resistance protein (BCRP) and multidrug resistance associated proteins (MRP) (Becker *et al.,* 2007; Dey *et al.,* 2003) have been reported for human cell culture models in comparison to animal cornea. These proteins can strongly influence the transport of substances through the epithelium (Dey and Mitra, 2004). Several substances have been identified as MDR1 substrates, such as daunorubicin (Shtil *et al.,* 2000), steroids, e.g. dexamethasone and hydrocortisone (Ueda *et al.,* 1992), and protease inhibitors, e.g. saquinavir (Huisman *et al.,* 2003) among others. The mRNA and functional expression of the MDR1 protein have been identified in *ex vivo* rabbit cornea (Dey *et al.,* 2003; Verstraelen and Reichl, 2013) as well as in *in vivo* rabbit cornea (Dey and Mitra, 2004). In addition, a weak mRNA expression of BCRP has been found in *ex vivo* rabbit cornea (Verstraelen and Reichl, 2013). Moreover, Verstraelen and Reichl have recently investigated the mRNA and protein expression as well as the functionality of some transporter proteins (MDR1, BCRP) with the HC construct (Verstraelen and Reichl, 2013). They have found that the HC construct exhibits mRNA and functional expression of BCRP but not of MDR1. Moreover, identical results have been reported for cultured primary rabbit corneal epithelial cells (Dey *et al.,* 2003; Kawazu *et al.,* 2003). All evidence indicates that the expression pattern is quite different for human and animal cornea and that some transport proteins could play an important role in the permeation of substances through the corneal barrier. Even though characterization experiments regarding transport proteins were out of scope of the current study, the comparison of the P_{app} rates obtained with the different substances showed that there was a difference in the permeability of the HC construct and *ex vivo* rabbit cornea. In further studies, transport experiments should be conducted with specific substrates and inhibitors in order to determine the functional expression of transport proteins, in particular SLC transporters, in the HC construct.

The comparison of other cell culture models, such as Caco-2 cell model and cell-based skin models, with the respective native tissues has revealed that they differ with regard to the permeability values. In the case of the Caco-2 cell model, it has been demonstrated that the permeation rate of drug substances correlated well with the intestinal absorption in humans for substances, which diffuse passively (Artursson and Karlsson, 1991). Nevertheless, the permeability of the Caco-2 cell model can be lower or higher than that of the human intestinal epithelium to substances being substrates of active transport proteins (Artursson *et al.,* 2001; Sun *et al.,* 2002; Lennernäs, 2007). The authors concluded that these differences in the permeability are mainly caused by the differences in the expression levels of drug-metabolizing enzymes and active transport proteins between the Caco-2 model and human

tissue. With regard to skin models, the permeability values reported for different drug substances have indicated that the permeability of *in vitro* skin models is significantly higher than that of *ex vivo* human skin. The different morphological structures, protein biding capacity, and the lack of lipids are the main reasons for this increased permeability of reconstructed skin models (Schäfer-Korting *et al.,* 2008; Netzlaff *et al.,* 2007).

Comparing the permeability of the HC construct with that of *ex vivo* human cornea indicated a high degree of similarity. The P_{app} values of five substances were compared and the correlation coefficient obtained from the linear regression demonstrates that the data obtained with the HC construct correlate well with the data previously reported for *ex vivo* human cornea. Moreover, the slope factor (0.94) shows that the permeation rates of hydrophilic and lipophilic substances achieved with the HC construct is expected to be almost identical to those of human cornea. Thus, these results show that the data obtained with the HC construct correspond better to those obtained with *ex vivo* human cornea than those achieved with rabbit cornea. This can be explained by the fact that the morphological characteristics of the HC construct and the human cornea are equivalent. The main advantage of an *in vitro* cornea model, which is based on human cells and equivalent to the human cornea is that the transferability of experimental results is more reliable than that of animal models since the results vary considerably between the species used (Hahne and Reichl*,* 2011; Becker *et al.,* 2008; Van Eyk *et al.,* 2009).

Despite of the good correlation between the permeability of the HC construct and data previously reported for *ex vivo* human cornea, it is premature to conclude that the HC construct can completely replace experiments using *ex vivo* animal tissue or *ex vivo* human tissue to investigate drug permeability. This is because the comparison of more than five substances with different physicochemical properties is required to determine whether the permeability of the HC construct and *ex vivo* human cornea is equivalent. Furthermore, the permeability data usually obtained with isolated human cornea have to be interpreted with caution for the following reasons: 1) human tissue used for experimental purposes is mostly not suitable for transplantation due to infectious diseases, e.g. hepatitis B (Reichl *et al.,* 2005) or cell degeneration, 2) the origin of the tissue is partly unknown, 3) human corneas are stored for an unknown period of time, which can affected the tissue quality. In addition, the transport proteins of the human cornea have been poorly investigated and the results reported to date by different research groups appear to be contradictory, which is probably due to the poor availability of suitable human tissue for such experiments (Becker *et al.,* 2007; Zhang *et al.,* 2008; Xiang *et al.,* 2009; Verstraelen and Reichl*,* 2013). From my point of view, more experiments need to be carried out with suitable human donor corneas in order to evaluate the transferability of data obtained with the HC construct to human situation. For

these reasons, experiments conducted with isolated corneas of animal origin, in particular of rabbits, remain to be best option to compare the results achieved with the HC construct in order to finally obtain a recognized *in vitro* corneal model that can be used by laboratories to investigate corneal drug absorption. In addition, formulation factors that influence the permeability of substances, such as pH, osmolality, or excipients (e.g. EDTA, preservatives) of ophthalmic formulations, also need to be further investigated and the results have to be compared with native tissue for the purpose of evaluating the suitability of the HC construct for drug permeability studies. The effect of these formulation factors is discussed in the following section.

5.1.3. Effect of formulation parameters and excipients on the barrier function of the HC construct

In order to determine the usefulness of the HC construct in the development of new formulations, the effect of different buffer solutions at different pH values ranging from pH 4.5 to 8.0 (the solutions contained either Na-FLU or mannitol) and with osmolalities ranging from 178 to 432 $mOsmol \cdot kg^{-1}$ (the solutions contained Na-FLU) on the epithelial integrity and corneal permeability of the HC construct was evaluated.

Within the scope of the current study it was observed that buffer solutions at pH values ranging from 5.0 to 8.0 did not affect the tightness of the HC construct for a period of time of 390 min. Furthermore, it was noted that these pH values changed the corneal permeability of the HC construct to the ionizable hydrophilic substance Na-FLU but not to the non-ionizable hydrophilic substance mannitol. In contrast, only the citrate buffer at pH 4.5 containing Na-FLU affected the integrity of the *in vitro* epithelium of the HC construct and increased the permeability to Na-FLU, whereas the same buffer solution containing mannitol affected the integrity of the HC construct to a lesser degree.

This is the first *in vitro* study, which has investigated in detail the effect of pH of different buffer solutions on epithelial integrity by determining changes of TEER of the HC construct. Unfortunately, the TEER values cannot directly be compared with those reported for *ex vivo* rabbit cornea. Despite of this limitation, the data obtained within the scope of the present study are in accordance with previous studies, which investigated the effect of different pH values on *ex vivo* rabbit and goat cornea. Regarding *ex vivo* rabbit cornea, changes in the integrity of the superficial corneal epithelial cells were evaluated in permeability studies with hydrophilic substances, e.g. mannitol (non-ionizable substance), and lipophilic substances, e.g. progesterone dissolved in a glutathione bicarbonated buffer solution. The results of these experiments indicated that the integrity of *ex vivo* rabbit cornea is not affected at pH values in the range from 5.0 to 7.7 since the P_{app} values achieved with mannitol and progesterone were identical (Suhonen *et al.,* 1998). Furthermore, Majumdar and co-workers evaluated the effect of different pH values on *ex vivo* goat cornea by measuring the

percentage of corneal hydration. The normal hydration level of the cornea lies between 75 and 80% (Rathore and Majumdar, 2006). This research group associated changes in the corneal tissue with an increase of the corneal hydration level. They reported that an aqueous solution containing moxifloxacin formulated at pH ranging between 5.5 and 7.2 did not affect the corneal integrity since the level of corneal hydration did not exceed 80% (Pawar and Majumdar, 2006). In short, the evidence mentioned above suggests that the integrity of *ex vivo* animal cornea is not affected when the pH of the buffer solution is raised from 5.0 to 7.7. Thus, the data obtained in the current study demonstrates that the HC construct can tolerate a change in the pH range similar to that reported for *ex vivo* animal cornea. In addition, in the current study, it has been observed that the pH-related effect on the integrity of the *in vitro* epithelium of the HC construct is dependent on the composition of the buffer solution. Similar results have also been reported for other corneal cell culture models. Tsai *et al.* have demonstrated that neither the vitality nor the TEER of corneal epithelial cells are significantly affected by the application of the culture medium at pH 5.0 (without any supplement) to the surface of the cultured human corneal epithelial cells (HCE cell line) (Tsai *et al*., 2010). In addition, another toxicity study conducted with pRCE cells has shown that a combination of citrate-acetate buffer or citrate-borate buffer at pH equal to or lower than 5.0 has a stronger effect on the corneal integrity than the phosphate-buffered solution (Dale and James, 1991a). These results are in agreement with the observations made with the HC construct in the current investigation in which the most pronounced effect was noted following the application of the citrate buffer at pH 4.5 containing Na-FLU. In contrast, the citrate buffer at pH 4.5 containing mannitol had a much lower impact. This can be explained by the fact that excipients such as mannitol reduce the toxic effect of preservatives (e.g. benzalkonium chloride) on the superficial corneal epithelium of *ex vivo* rabbit cornea and of the HCE-T epithelial cell model (Mohamed *et al.,* 2016; Nagai *et al.,* 2011).

No specification that defines which pH level can be tolerated by the corneal tissue in *ex vivo* or *in vitro* studies has yet been established. In further studies, the experiments conducted in the current investigation should be complemented with toxicological experiments and the results should be compared to those obtained with *ex vivo* rabbit cornea in order to define a guideline, which contains acceptable pH thresholds at which ophthalmic medications should be formulated in order to avoid any significant modification to the superficial corneal epithelium. Moreover, the current study shows that the corneal permeability of the HC construct can be influenced by the pH and composition of the buffer solution used. Moreover, it has been demonstrated that changes in the corneal permeability of the HC construct are associated with the physicochemical properties of substances. In the case of the hydrophilic permeation marker Na-FLU, which is an ionizable substance, the permeability of the HC construct is increased when the pH of the buffer solution is reduced or its composition is

modified. In contrast, neither changes in the pH nor the composition of the buffer solution altered the permeability of the HC construct to mannitol. All evidence obtained from the literature indicates that the permeability properties of *ex vivo* rabbit cornea or other *ex vivo* animal cornea (e.g. sheep, goat) can be altered by changes in the pH and composition of buffer solutions if they affect the lipophilicity and the solubility of substances. Thus, it has been concluded that these changes in the corneal permeability of *ex vivo* rabbit cornea is dependent on the physicochemical properties of the evaluated substances (Cherng-Chyi and Lidgate, 1986; Fu and Lidgate, 1986; Rojanasakul and Robinson, 1989; Suhonen *et al.,* 1998; Anand and Mitra, 2002; Fuchsjäger-Mayrl *et al.,* 2002; Ahuja *et al.,* 2006).

Regarding the effect of the osmolalities, only the non-isotonic solutions at 178 mOsmol·kg^{-1} and 432 mOsmol·kg^{-1} altered the barrier properties of the HC construct after 390 min of incubation. Based on these experimental results it can be concluded that the long-term application of non-isotonic solutions leads to changes in the structures of the superficial corneal epithelium of the HC construct. Even though experiments with *ex vivo* rabbit cornea, which determine the effect of non-isotonic solutions on the TEER have not yet been carried out up to now, the data of the current investigation can be compared with those obtained in investigations with other *in vitro* corneal models. In this context, toxicological studies with non-isotonic solutions at osmolalities ranging from 100 to 1000 mOsmol·kg-1 were conducted with pRCE cells (Dale and James, 1991b). This group reported that pRCE cells are only able to tolerate non-isotonic solutions at osmolalities ranging from 100 to 600 mOsmol·kg^{-1} after 64 min and 128 min of application. Thus, the effect seen in the HC construct is consistent with that reported in the literature. Moreover, the changes of the integrity (TEER) of the HC construct observed after 390 min did not affect its permeability to Na-FLU. It has been reported that only non-isotonic solutions at osmolalities lower than or equal to 83 mOsmol·kg^{-1} or higher than or equal to 582 mOsmol·kg^{-1} can influence the permeability of *ex vivo* rabbit cornea (Ashton *et al.,* 1991). In further studies, the effect of highly non-isotonic solutions (e.g. 80, 100 and 600 mOsmol·kg^{-1}) could be evaluated in order to determine whether the corneal permeability of the HC construct is also affected by these osmolalities as has previously been reported for *ex vivo* rabbit cornea. Moreover, it has been described that the instillation of hypertonic solutions into the eye intensifies the precorneal loss process (e.g. blinking, lacrimation and tear turnover) resulting in a reduction of the precorneal residence time of the drug and thereby decreases its ocular bioavailability (Podder *et al.,* 1992; Carlfors *et al.,* 1998). In contrast, it has been suggested that the application of hypotonic solutions does not cause lacrimation (Conrad *et al.,* 1978), whereby the residence time of the drug on the precorneal surface is prolonged resulting in an increase of the drug permeation through the corneal barrier (Podder *et al.,* 1992; Carlfors *et al.,* 1998).

Other authors have proposed that non-isotonic solutions produce an osmotic gradient between the tear film and the surrounding tissue that enhances the permeation of the drug through the cornea (Malhotra and Majumdar, 2001). Thus, it can be suggested that these factors play an important role under *in vivo* but not *in vitro* conditions. In further studies, the experimental methodology needs to be refined in order to be able to imitate these important precorneal conditions, which influence *in vivo* the bioavailability of ocular drugs.

5.1.4. Preservative effects on the HC construct

Ophthalmic medications are usually formulated with preservatives and chelating agents in order to conserve their microbial quality during use. However, it has been reported that the administration of preserved ophthalmic medications into the eye results in alterations of the integrity and permeability of the corneal tissue (Baudouin *et al.,* 2010). Thus, the current investigation evaluates the effect of preservatives and the chelating agent EDTA on the barrier function of the HC construct.

Within the scope of the current study, it was found that EDTA and preservatives (BAC, MP, Thio, and PQ-1) change the permeability of the HC construct in a time-concentration dependent manner. The effect that has been observed for EDTA and BAC, MP, Thio, and PQ-1 is also related to their toxic properties. In contrast, the preservatives Ce, Purite®, sofZia® have not affected the permeability of the HC construct. The few data available in the literature demonstrate that the effect of MP, Thio, Ce and the alternative preservatives (PQ-1, Purite®, sofZia®) on the native corneal epithelium has been poorly investigated. Thus, this study aims to provide additional information about the effect of these excipients, which needs to be considered during the development of new ophthalmic medications. In contrast, the effect of the foremost used preservative BAC and the ophthalmic excipient EDTA on the native tissue has been intensively investigated, thus it was possible to assess to which extent the data obtained with the HC construct are transferable to native tissue. For example, *in vivo* animal experiments demonstrated that BAC concentrations higher than or equal to 0.005% led to a rapid TEER reduction of the rabbit cornea after 1 min of application. In contrast, BAC concentrations lower than or equal to 0.002% did not decrease the TEER recorded for *in vivo* rabbit cornea (Uematsu *et al.,* 2007; Kusano *et al.,* 2010).

However, the *in vivo* effect of these BAC concentrations (0.001% and 0.002%) on the corneal surface is certainly questionable due the following reasons: Firstly, the ophthalmic solution is removed from the corneal surface after 2 to 4 min in humans (Malhotra and Majumdart, 2001; Urtti, 2006). Secondly, some ocular diseases require the application of the ophthalmic medication more than once per day within the scope of a long-term treatment, which could lead to the accumulation of BAC in the corneal epithelium and the anterior stroma (Green and Chapman, 1986; Noecker, 2001; Chen *et al.,* 2014). In contrast, it has been reported for *ex vivo* rabbit cornea that 0.001% and 0.002% BAC reduced the TEER by

more than 50% following a time of between 60 and 80 min of application. In addition, the application of 0.01% and 0.02% BAC on the surface of *ex vivo* rabbit cornea resulted in a reduction of the TEER that was higher than that noted for 0.002% BAC (Chetoni *et al.,* 2003; Nakamura *et al.,* 2007). All these toxicological experiments have demonstrated that the reduction of the TEER *in vivo* and *in vitro* correlated with the cytotoxic effect of BAC. These observations indicate that concentrations higher than or equal to 0.001% BAC can affect the TEER of *ex vivo* rabbit cornea. Thus, the data obtained with the HC construct are in agreement with those previously reported for *ex vivo* rabbit cornea. In addition, toxicological experiments with pure corneal epithelial cell culture models have demonstrated that 0.001% and 0.0025% BAC reduces the percentage of living cells (HCE cells) to approximately 60% and 40% after 20 min of application. This effect was even higher after 60 min of application (Guzman-Aranguez *et al.,* 2014). Burgalassi *et al.* have shown that 0.001% BAC decreases the percentage of living cells (HCE and RCE cells) to 15% after 15 min of application (Burgalassi *et al.,* 2001). Other research group has also found that 0.0025% BAC decreases the percentage of living cells (HCE cells) to approximately 55% after 60 min of application (Epstein *et al.,* 2009). Against this background, it can also be concluded that the effect of BAC on other *in vitro* epithelial cells is related to its cytotoxic properties, concentration as well as the exposure time. Moreover, the results of the current study demonstrate that BAC concentrations, which have not affected the tightness of the *in vitro* corneal epithelium have not changed the corneal permeability of the HC construct to neither hydrophilic nor lipophilic substances. In contrast, BAC concentrations, which have decreased the epithelial tightness have also led to a significant change in the corneal permeability of the HC construct to the hydrophilic substance Na-FLU. These data are in accordance with those obtained in previously described permeability studies, which assessed the effect of BAC on the permeability of *ex vivo* rabbit cornea to hydrophilic substances (López Bernal and Ubels, 2001; Ubels *et al.,* 2004; Nakamura *et al.,* 2007; Majumdar *et al.,* 2008). However, the effect of such concentrations of BAC or other preservatives, which cause a reduction in the tightness of the HC construct has not been evaluated for non-hydrophilic substances (e.g. bimatoprost). Despite of this limitation, permeability studies previously carried out with lipophilic drugs demonstrated that such BAC concentrations (e.g. 0.01 or 0.02%) increased the permeability of the native tissue to lipophilic drugs (Camber and Edman, 1987; Ashton *et al.,* 1991; Maccarey and Edelhauser, 2007; Chang *et al.,* 2012). Finally, the data obtained in the current study indicate that the HC construct is suitable to investigate the effect of ophthalmic excipients since they are comparable to those reported for *ex vivo* rabbit cornea. The effect of other kinds of excipients (e.g. cyclodextrins, mucoadhesive polymers) and ophthalmic formulations remains to be evaluated and compared to *ex vivo* native tissue within the scope of further studies. Moreover, the dynamic processes, which take place

under *in vivo* conditions, e.g. the blinking of the eye, the tear turnover and the dilution of the drug by the tear film, also need to be simulated under experimental conditions in order to ensure that the data obtained with the HC construct can be transferred to the situation *in vivo*.

6. Summary and outlook

The present study focused on the method transfer and further characterization of an *in vitro* human Hemicornea model that has been developed by the Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Germany. The first step of the present investigation was to establish the HC construct at the biotech laboratory ACB by transferring standardized protocols that have been developed and provided by TUBS. The HC construct, which is based on two immortalized human corneal cell lines, is used as an alternative to *in vivo* methods employed for drug absorption studies. The successful protocol transfer was demonstrated on the basis of the barrier properties of the HC construct (TEER and P_{app}), which fulfilled acceptance criteria previously defined by TUBS. Furthermore, after successful transfer, additional quality criteria for further HC batches were defined at ACB and applied within the scope of this investigation. A minimum TEER value of 400 Ohm·cm² was required to achieve P_{app} values < $1 \cdot 10^{-6}$ cm·s^{-1} for the hydrophilic permeation marker Na-FLU.

In a second step, the intra- and interlaboratory reproducibility of the HC construct was assessed, which is critical requirement of *in vitro* alternative models in order to be accepted by the respective authorities. The prevalidation phase was conducted at three different laboratories (TUBS, ACB and UKE) by performing permeability experiments with Na-FLU, FD-4, rhodamine B and four ophthalmic drugs, namely aciclovir, bimatoprost, dexamethasone and timolol maleate. The resulting data demonstrated that the intra- and interlaboratory reproducibility of permeability experiments with the HC construct is distinctly higher than for experiments using *ex vivo* rabbit cornea. Additional *in vitro* permeability studies were conducted with substances having a wide range of different physicochemical properties and compared to those previously reported for native corneal tissue of rabbit and human origin. The data indicated that the permeability of the HC construct is quite similar to that of rabbit and nearly equivalent to that of human cornea.

With regard to a widespread use of the HC construct in preclinical drug development, in a third step, the influence of formulation parameters, such as the pH, osmolality and excipients (e.g. EDTA and preservatives) on the barrier properties of the HC construct was also investigated. The results demonstrated that the barrier properties of the HC construct are not influenced in a wide range of pH and osmolality. Only extreme values of pH and osmolality resulted in alteration of barrier properties and permeability of the HC construct. Furthermore, results from experiments studying the influence of EDTA and preservatives showed that the impact of most excipients is both, concentration as well as time dependent, and in general based on the cytotoxic potential of these substances. However, comparison with reports from

the literature including similar studies on *in vivo* or *ex vivo* animal tissue disclosed that the results obtained with the HC construct are very similar to that of animal cornea.

In conclusion, it can be stated that the HC construct can be successfully transferred in other laboratories. The HC construct can be used to predict the corneal permeability to ophthalmic drugs and to screen excipients and formulations for ophthalmic drugs. Despite some limitations, the HC construct is a promising *in vitro* model to reduce animal testing in development of ophthalmic drugs.

Nevertheless, some points should be considered to improve the cultivation of HC construct and the significance of drug absorptions studies. In future investigations, the culture conditions of the HC construct have to be refined in order to increase the stability of its TEER level for a period of time longer than 11 days. In addition, excipients, such as preservatives, should be evaluated over an incubation time no longer than 5 or 10 min. Such short incubation time represents the common precorneal residence time of ophthalmic formulations and should result in more significant outcomes in drug development. Moreover, these studies can be complemented with toxicological experiments, i.e. WST-1 or LDH assays. Furthermore, drug permeability studies should be conducted under simulated *in vivo* physiological conditions (e.g. the use of tear fluid and the simulation of precorneal loss factors) in order to obtain more accurate information with regard to the ocular pharmacokinetics (absorption and desorption rates) and the bioavailability of ophthalmic drugs.

7. References

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- Investigation of the physiological role of Ca^{2+} channel β subunits in the modulation of the synaptic plasticity using continental and autaptic hippocampal neurons from mice

10/2009 – 10/2014: Across Barriers GmbH, Saarbrücken
Scientist, Ph.D. student

- Transfer, prevalidation and further investigation of a 3D Human cornea model as an alternative method for animal testing, which is based on a human cornea epithelial cell line (HCE-T) and a human cornea keratocyte cell line (HCK)
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04/2003 – 02/2004: PROCAPS S.A Barranquilla – Columbia

Document technician

- Organisation and actualisation of the technical documentation of pharmaceutical products to obtain sanitary registration of health products by the National Institute for Medicine and Food Surveillance (INVIMA)

Education

02/1998 – 12/2003: Studies at the Pharmacy & Chemistry department at the University of Cartagena - Columbia

- "Diploma Químico Pharmaceutico" Degree in Chemical Pharmacy (equivalent to the German "Diplom")
- Subject of final thesis: Chemical Study of Hexanoic Extract of the Colombian Caribbean Marine Sponge (Xetospongia subtriangularis)

01/1992 – 11/1997: Secondary School at Military School Abolsure Cartagena – Columbia

01/1987 - 12/1991: Elementary school at Institute Pestalozzi Cartagena – Columbia

Languages

Spanish (native speaker), English(fluent), German(fluent)

Computer Skills

Microsoft Office 2010, Word (excellent), Power Point (excellent), Excel (excellent) and Outlook (good)

Microcal Origin version 6.0 / 8.6, SigmaPlot version 8.0

HPLC-MS software skills: Empower 2 (excellent), ChemStation (excellent), MassLynx (excellent)

Patch Clamp: Heka

Publications

- Prevalidation of a human cornea construct as an alternative to animal corneas for in vitro drug absorption studies. Hahne M, Zorn-Kruppa M, Guzman G, Brandner JM, Haltner-Ukomado E, Wätzig H, Reichl S. J Pharm Sci. 2012 Aug;101(8):2976-88
- TRPC5 is a Ca^{2+} activated channel functionally coupled to Ca^{2+}-selective ion channels. Gross SA, Guzman GA, Wissenbach U, Philipp SE, Zhu MX, Bruns D, Cavalié A. J Biol Chem. 2009 Dec 4;284(49):34423-32